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Abstract

Variation in the calpain-10 gene has been linked to a three-fold increased risk for type 2 diabetes in Pima Indian and some European populations. Furthermore, reduced skeletal muscle expression of calpain-10 is associated with reduced insulin mediated glucose disposal and carbohydrate oxidation. The skeletal muscle specific calpain-3 plays a key role in skeletal muscle integrity and has also been linked to insulin resistance in humans and rodents.

The major aims of this thesis were to 1) investigate the hypothesis that alterations in insulin sensitivity in healthy humans would lead to significant changes in the mRNA and protein expression of calpain-10 and -3, 2) investigate the effect of hyperinsulinaemia and lipid availability on calpain-10 and -3 expression, 3) further address the role of genetic variation in the calpain-10 gene on glucose utilisation in humans and finally 4) investigate the expression of calpain-10 in skeletal muscle of type 2 diabetic patients.

The studies in this thesis show for the first time that insulin resistance as a result of short term fasting or high fat availability is not associated with changes in calpain-10 and -3 mRNA and protein expression, providing evidence against an adaptive role for these genes in the development of fasting- and lipid-induced insulin resistance.

Analysis of human skeletal muscle protein demonstrated, also for the first time, the expression of multiple calpain-10 isoforms and revealed that calpain-10 is highly localised to the nucleus and plasma membrane. Whilst short-term physiological hyperinsulinaemia was found to negatively regulate calpain-10 mRNA and protein, acute exercise did not lead to changes in calpain-10 and -3 expression. Genetic variation in the calpain-10 gene, previously linked to type 2 diabetes, was not associated with insulin mediated glucose disposal or rates of carbohydrate and fat oxidation in healthy humans. Finally, total calpain-10 protein expression was found to be lower in the skeletal muscle of type 2 diabetic patients when compared to age matched endurance trained, but not sedentary, control subjects. These findings are highly relevant for studies into the underlying genetic causes of diabetes and for the ongoing search for diabetes linked genes.

Publications

Abstracts

L. Norton, K. Chokkalingam, K. Jewell, T. Parr, K. Tsintzas (2006). Insulin, but not lipid availability, regulates calpain-10 at the transcriptional level in human skeletal muscle (<http://www.eb2006-online.com>). 2006 Experimental Biology meeting abstracts. *FASEB J*, 20, Abstract **LB144**

L. Norton, K. Chokkalingam, K. Jewell, T. Parr and K. Tsintzas (2006). Effect of exercise on the expression of metabolic genes involved in glucose transport and oxidation in human skeletal muscle. Physiological Society meeting abstracts. *J Physiol*, Abstract **C56**

Papers

L. Norton, T. Parr, R. Bardsley, H. Ye and K. Tsintzas (2006). Characterization of GLUT4 and calpain expression in healthy human skeletal muscle during fasting and refeeding. *Acta Physiol* **189 (3)**, 233-240.

K. Chokkalingam, K. Jewell, L. Norton, J. Littlewood, L. J. C. vanLoon, P. Mansell, I. A. Macdonald and K. Tsintzas (2006). High fat/low carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates non-oxidative glucose disposal in humans: an important role for skeletal muscle PDK4. *JCEM* **92 (1)**, 284-292.

Declaration

All of the procedures presented in this thesis have been performed by myself with the following exceptions:

Dr Kamal Chokkalingam performed all cannulations, infusions, insulin clamps and muscle biopsies throughout this thesis except in **chapter 3**, where all medical procedures were performed by Dr David Laithwaite prior to the commencement of the authors' PhD studies. A number of biochemical assays were also performed by Dr Kamal Chokkalingam and these have been clearly indicated in the text. The determination of blood ketones in **chapter 4** was performed by Sally Cordon and this is also indicated in the text. Glucose tracer analysis and patient recruitment and investigation in **chapters 4 and 6**, respectively, were performed by Dr L.J. van Loon at Maastricht University, The Netherlands as indicated in the text.

I hereby declare that the present thesis has been composed by myself and that the work, of which this is a record, has been performed by myself, except where assistance has been acknowledged. No part of this thesis has been submitted to any previous application for a higher degree. All sources of information have been specifically referenced.

Luke Norton

17th October 2006

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Abbreviations

°C	Celsius
4EBP1	Eukaryotic translation initiation factor 4E binding protein-1
ADP	Adenosine diphosphate
AMPK	Adenosine monophosphate kinase
ANOVA	Analysis of variance
AS160	Rab-GTPase-activating protein AS160
Asn	Asparagine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca ²⁺	Calcium ion
CaMKII	Calcium/calmodulin dependent protein kinase II
CAPN10	Calpain-10 gene
CAPN3	Calpain-3 gene
cDNA	Complementary DNA
CHO	Carbohydrate
cM	Centimorgan
CON	Control
COX	Carbohydrate oxidation
CPT1	Carnitine palmitoyltransferase-I
Cys	Cysteine
d	Days
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECL/ECL+	Enhanced chemiluminescence/plus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EMSA	Electromobility shift assay
ES	Embryonic stem (cell)
EST	Expressed sequence tag
EtBr	Ethidium bromide
EX	Exercise
FFA	Free fatty acid
FM	Fat mass

FOX	Fat oxidation
FOXO	Forkhead transcription factor
g	Gram
GIR	Glucose infusion rate
GLUT	Glucose transporter
Gly	Glycine
GS	Glycogen synthase
GSK	Glycogen synthase kinase
HF	High fat
HGO	Hepatic glucose output
His	Histidine
HK	Hexokinase
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IGT	Impaired glucose tolerance
Insr	Insulin receptor gene
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IR	Insulin receptor
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
I κ B α	Inhibitor κ B-alpha
kDa	Kilo Dalton
K _m	Michaelis constant
KO	Knock-out
LB	Lauria-Bertani media
LGMD2A	Limb girdle muscular dystrophy type 2A
LIPID	Intralipid
MAPK	Mitogen activated protein kinase
MEF2	Myocyte enhancer factor-2
MIRKO	Muscle insulin receptor knock-out
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NF- κ B	Nuclear Factor κ B
NGT	Normal glucose tolerance
NOD	Non oxidative glucose disposal
OGTT	Oral glucose tolerance test
ORF	Open reading frame
P13K	Phosphatidylinositol 3-kinase
p70S6K	p70 ribosomal protein S6 kinase

p94	Calpain-3
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PDK	3-phosphoinositide-dependent protein kinase
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
PH	Pleckstrin homology domain
PI3K	Phosphoinositide-3 kinase
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKB/AKT	Protein kinase B
PKC	Protein kinase C
PP1	Protein phosphatase-1
PTB	Phosphotyrosine binding domain
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylidene fluoride membrane
Ra	Rate of glucose appearance
Rd	Rate of glucose disappearance
RER	Resting energy expenditure
RMR	Resting metabolic rate
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
s.e.m	Standard error of the mean
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SH2	SRC homology-2 protein
SNAP	N-ethylmaleimide-sensitive attachment protein
SNARE	Membrane-associated SNAP protein
SNP	Single nucleotide polymorphism
TAE	Tris acetate EDTA
TBS-T	Tris buffered saline with Tween
TE	Tris-EDTA buffer
Tris	2 amino-2-(hydroxymethyl)-propane-1,3-diol
TSC	Tuberous sclerosis complex-2
v/v	Volume per volume
VAMP	Vesicle associated membrane protein
vol	Volumes
w/v	Weight per volume
α	Alpha

β	Beta
γ	Gamma
δ	Delta
δ	Delta
ε	Epsilon
ζ	Zeta
η	Eta
θ	Theta
ι	Iota
λ	Lambda

1 Introduction

1.1 In perspective

Diabetes is characterised by abnormally high levels of sugar (glucose) in the blood. Under normal physiological conditions, a rise in blood glucose triggers the release of the anabolic hormone insulin from the pancreas. The major role of insulin is to stimulate muscle and fat cells to remove excess glucose from the blood, and to stimulate the liver to metabolise glucose, thereby causing the blood sugar level to return to normal fasting values. In people with diabetes, blood sugar levels remain elevated. This may be because insulin is not being produced, is not made at sufficient levels, or is not as effective as it should be. The two major forms of diabetes are type 1 diabetes (5%), which is primarily an autoimmune disorder, and type 2 diabetes (95%), which is commonly associated with obesity.

Type 2 diabetes is the most common disease in the world (King and Rewers, 1993; King et al., 1998; Mokdad et al., 2001a; Mokdad et al., 2001b) and in the United States alone, it is a leading cause of blindness (Klein et al., 1995; Porta and Allione, 2004), kidney disease (Cowie et al., 1989) and nontraumatic loss of limb (Reiber and Raugi, 2005). The associated health care costs in the United States are estimated to be in excess of \$130 billion a year. In the UK, approximately 1.6 million

people have been diagnosed with diabetes and the associated costs to the National Health Service are approximately £5.2 billion a year. On a wider scale, type 2 diabetes is set to become a global pandemic with an estimated 300 million individuals affected by the year 2025 (King et al., 1998).

Despite the fact that type 2 diabetes has been known for approximately 2000 years, the precise mechanisms underlying the disease remain largely unknown. The pathogenesis of type 2 diabetes is undoubtedly complex but it is clear from studies on twins that genetic predisposition is an important factor in the development of the disease (Froguel et al., 1993; Guillausseau et al., 1997; Medici et al., 1999; Newman et al., 1987). However, the pattern of inheritance for the vast majority of type 2 diabetic patients reflects varying penetrance or a polygenic nature.

1.2 Insulin and the regulation of blood glucose

1.2.1 Insulin signalling

Insulin is essential for maintaining normal physiological levels of glucose in the blood, or euglycaemia, and is released from the β -cells of the pancreas in response to a glucose load. Insulin exerts its effects by binding to and activating the membrane bound insulin receptor (IR) tyrosine kinase. Insulin receptors are widely expressed throughout the human body, but insulin exerts most of its effects through three major target tissues - skeletal muscle, adipose tissue and the liver.

In the skeletal muscle, which is the focus of this thesis, binding of insulin to the IR activates a cascade of intracellular events that leads to the removal of glucose from the blood into the cell. In the presence of insulin, the insulin receptor phosphorylates insulin receptor substrate proteins (IRS), which are linked to the activation of the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway, which is responsible for a large majority of the metabolic actions of insulin.

1.2.1.1 The IR and IRS proteins

The IR is the first point of contact between circulating insulin and an insulin response. The importance of the IR has been demonstrated through the use of knock-out mice. Targeted disruption of the insulin receptor gene (*Insr*) in mice resulted in marked hyperglycaemia and hyperinsulinaemia, ketoacidosis and growth retardation and finally death seven days after birth (Joshi et al., 1996). In humans there have been just four case reports of homozygous *Insr* mutations (Tritos and Mantzoros, 1998). Skeletal muscle specific IR knock out (MIRKO) leads to impaired insulin signalling and decreased insulin dependent glucose transport but not whole body insulin resistance (Bruning et al., 1998).

The insulin receptor satellite proteins known as IRS are activated by phosphorylation and mediate binding of intracellular effectors. To date, six IRS proteins have been identified and it is IRS1 and IRS2 which appear to be the most important. The IRS proteins associate with the IR via N-terminal pleckstrin-homology domains (PH) and phosphotyrosine-binding domains (PTB). They associate with intracellular molecules containing Src-homology-2 domains (SH2) following the tyrosine phosphorylation of up to twenty sites located in the central and C-terminal regions of IRS proteins. Perhaps the best-studied SH2 protein is the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (Van Obberghen et al., 2001, Taniguchi et al., 2006).

1.2.1.2 Phosphatidylinositol 3-kinase (PI3K)

Phosphorylation and activation of the IR and IRS leads to the activation of PI3K. The importance of PI3K is clear as transfection with dominant-negative PI3K blocks many of insulin's actions (Cheatham et al., 1994). The PI3K enzyme consists of a regulatory and catalytic subunit; the activation of the catalytic subunit depends on the interaction of the two SH2 domains in the regulatory subunit with specific phosphotyrosine motifs in the IRS proteins (Shepherd et al., 1998). PI3K exerts its effects by catalysing the formation of the lipid second messenger Phosphatidylinositol (3,4,5)-triphosphate (PIP_3), which is able to bind and activate additional proteins via their PH domains (Shepherd et al., 1998). One of the most important group of proteins that bind to PIP_3 in this way is the AGC superfamily of serine/threonine protein kinases. The most critical of these proteins is the 3-phosphoinositide-dependent protein kinase 1 (PDK1), which is responsible for the activation of AKT/protein kinase B (PKB) (Alessi et al., 1997). AKT/PKB requires phosphorylation at two sites for its activation – Thr308 and Ser473; PDK1 enhances the activity of AKT/PKB by phosphorylation of Thr308 only (Alessi et al., 1997). Thus for full activation of AKT/PKB, it has been suggested that there is a PDK2 which phosphorylates Ser473. Indeed recent evidence suggests that this might be the rapamycin-insensitive companion of mTOR (rictor) - mammalian target of rapamycin (mTOR) complex (Sarbasov et al., 2005). The positive actions of PI3K can be negatively regulated at the level of PIP_3 by

phospholipid phosphatases, such as phosphatase and tensin homologue (PTEN) which dephosphorylates and inactivates PIP_3 (Maehama and Dixon, 1999).

1.2.1.3 AKT/PKB

AKT/PKB mediates most of the PI3K – dependent metabolic actions of insulin, through the phosphorylation of many substrates including other kinases, signalling proteins and transcription factors. One of the major roles of the AKT/PKB pathway is to stimulate glucose uptake into the cell and upregulate glycogen synthesis. AKT/PKB appears to regulate glucose uptake by phosphorylating and inhibiting the Rab-GTPase-activating protein AS160 (Kane et al., 2002; Sano et al., 2003). This may trigger the activation of Rab small GTPases that are important for the cytoskeletal re-organisation that is required for the translocation of the glucose transporter GLUT4 to the plasma membrane (see 1.2.1.4). Phosphorylation of glycogen synthase kinase-3 (GSK3) by AKT/PKB decreases its activity towards glycogen synthase and thereby increases glycogen synthesis (Frame and Cohen, 2001) (see 1.3.3). AKT/PKB also activates the mTOR pathway, possibly via phosphorylation and inhibition of tuberlin, or tuberous sclerosis complex-2 (TSC2), which is in complex with hamartin (TSC1) (Harris and Lawrence, 2003). The mTOR pathway regulates protein synthesis by phosphorylating the proteins p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic

translation initiation factor 4E binding protein-1 (short, 4EBP1) (Harris and Lawrence, 2003).

Finally, AKT/PKB regulates the expression of gluconeogenic and lipogenic enzymes by controlling the activity of the forkhead (FOX) class of transcription factors – a family of around 100 members, several of which may be critical for insulin action. For example, FOXO1 activates gluconeogenic genes in the liver and inhibits adipogenesis, actions which are inhibited by insulin through AKT/PKB mediated phosphorylation of FOXO1, which inhibits its transcriptional activity. FOXO1 may also have an important role in the regulation of carbohydrate and fat oxidation via actions on the pyruvate dehydrogenase complex (PDC) (1.3.4) (Taniguchi et al., 2006).

1.2.2 Insulin signalling and glucose uptake

Activation of the insulin signalling pathway through AKT/PKB leads to the translocation of intracellular glucose transporters to the plasma membrane where they facilitate glucose uptake into the target tissue. Whilst there are five major glucose transporters with distinct tissue distributions (Shepherd and Kahn, 1999; Joost et al., 2002; Bell et al., 1990), the most abundant in insulin sensitive tissues is GLUT4. GLUT4 has a glucose K_m of ~ 5 mM, which is close to the plasma glucose concentration of a healthy human. In skeletal muscle and adipose

tissue, the concentration of GLUT4 at the plasma membrane increases markedly in response to insulin.

In unstimulated muscle and adipose tissue, it is thought that GLUT4 constitutively cycles to and from the plasma membrane through slow endocytosis and fast exocytosis, and it is thought that insulin stimulates the exocytic arm and reduces the endocytic arm of this cycling (Bryant et al., 2002; Dugani and Klip, 2005). Whilst the exact mechanisms that lead to GLUT4 translocation following stimulation by insulin are not fully understood, it is currently thought that AKT/PKB (and possibly protein kinase C, 1.2.1.5) regulates GLUT4 translocation and glucose uptake by phosphorylating and inhibiting the Rab-GTPase-activating protein AS160 (Sano et al., 2003) as mentioned above. In addition to stimulating the translocation of GLUT4, insulin derived signals also facilitate the fusion of GLUT4 containing vesicles with the plasma membrane. AKT/PKB can target components of the vesicle – plasma membrane fusion machinery, which comprises the vesicle-associated membrane protein-2 (VAMP2), the *N*-ethylmaleimide-sensitive attachment protein-23 (SNAP23) and the membrane-associated SNAP protein (SNARE) syntaxin-4 as well as synip, tomosyn and munc18 that bind syntaxin4 to modulate the insulin dependent gain in membrane associated GLUT4 (Dugani and Klip, 2005).

1.2.3 Atypical protein kinase C (PKC) and glucose uptake

A number of PKC isoforms have been linked to the regulation of the insulin signalling pathway, both in a negative (see 1.3.1.4) and a positive fashion. The term protein kinase C (PKC) actually describes a family of protein kinases consisting of approximately 10 isozymes. They are commonly further subdivided into conventional (classical), novel and atypical forms. Conventional PKCs (cPKC) consist of α , β and γ isoforms and require calcium, diacylglycerol (DAG) and a phospholipid for activation. The novel PKCs (nPKC) consist of δ , ϵ , η and θ isoforms and require DAG but not calcium for their activation. In contrast, the atypical PKCs (aPKC) include ζ and λ isoforms but these do not require either calcium or DAG for activation and are similar to AKT/PKB, which require PIP3 and PDK1/PDK2 phosphorylation (Dey et al., 2006).

In addition to the phosphorylation and activation of AKT/PKB, it has been demonstrated that an additional target of PDK1 is PKC- ζ and - λ and there is now evidence to suggest that these aPKC isoforms serve as molecular switches that participate in turning on glucose transport responses during insulin action (Farese, 2002). The aPKCs exist in cells in a folded state and, upon activation by acidic lipids (e.g. PIP3), unfold and become active through a number of complementary mechanisms, including PDK1 phosphorylation, autophosphorylation and relief of auto-inhibition, suggesting that mechanism exist to activate

aPKC independent of phosphorylation and PDK1 (Farese, 2002). Following unfolding, aPKCs may become exposed to protease activity and are then converted to short lived, constitutively active M-type kinases (see 1.8.2.5).

Defects in insulin mediated aPKC activation have consistently been reported in the muscle and adipocyte of type 2 diabetic patients (Beeson et al., 2003; Kim et al., 2003) and in a number of animal models of insulin resistance (Standaert et al., 2002; Kanoh et al., 2001). Stable expression of kinase inactive aPKC in myotubes inhibits insulin mediated GLUT4 translocation, whereas overexpression of constitutively active aPKC mimics the action of insulin on GLUT4 translocation (Bandyopadhyay et al., 1997). Furthermore, embryonic stem (ES) cells which lack PKC- λ , and which are then differentiated to adipocytes, insulin fails to stimulate glucose transport (Bandyopadhyay et al., 2004). Together, these studies strongly suggest that aPKCs play an important role in glucose uptake/GLUT4 translocation in skeletal muscle and adipose tissue. There is currently no evidence to suggest that the conventional or novel PKC isoforms play a similar role in insulin stimulated glucose transport/GLUT4 translocation. Indeed, as mentioned above, they appear to be more relevant in states of insulin resistance (see 1.3.1.4).

1.2.4 Glucose phosphorylation

Once glucose has successfully entered the cell, isoenzymes of hexokinase (HK) catalyse the conversion of glucose to glucose-6-phosphate – essentially trapping glucose within the cell. HKI, HKII and HKIII are single chain peptides that have a number of properties in common, including a high affinity for glucose and inhibition by glucose-6-phosphate. HKII is expressed in insulin sensitive tissues (adipose and muscle tissue) whereas HKI is expressed in brain and erythrocytes. HKIV, also known as glucokinase, can be further subdivided into HKIVB, which is believed to be the glucose sensor in the β -cell, and HKIVL, which is important for hepatic glucose metabolism (Katzen et al., 1970; Wilson et al., 2003).

1.2.5 Glycogen metabolism – non oxidative glucose disposal

During rest, storage of glucose as glycogen is enhanced by insulin stimulation of glucose uptake. Following phosphorylation to glucose-6-phosphate (G-6-P) by HKII, glucose may enter glycolysis to produce ATP or be converted to glucose-1-phosphate for glycogen synthesis. Glycogen synthase (GS) is one of the key enzymes controlling the rate of muscle glycogen synthesis and utilises UDP-glucose to add glucose molecules by α 1 – 4 linkages – the rate limiting step in glycogen synthesis.

The activity of GS is controlled by covalent modification (phosphorylation/dephosphorylation), allosteric activation and enzyme translocation. The enzyme is phosphorylated on up to nine residues by several kinases, resulting in enzyme deactivation and decreased sensitivity to allosteric activators. These kinases include cAMP-dependent protein kinase (PKA), calmodulin-dependent kinases, adenosine monophosphate kinase (AMPK) and glycogen synthase kinase 3 (GSK3). Conversely, the dephosphorylation and activation of GS is controlled by protein phosphatase 1 (PP1). These enzymes themselves are also closely regulated: GSK3 can be negatively regulated by phosphorylation by AKT/PKB following stimulation by insulin and positively regulated by cAMP following adrenergic stimulation. Similarly, PP1 can be upregulated by insulin via complex mechanisms leading to dephosphorylation and activation of GS. The relative contribution of GSK3 and PP1 to GS activity is not clear but recent reports on transgenic mice have questioned the ability of insulin to activate GS via PP1 (Suzuki et al., 2001; Walker et al., 2000).

Alternative mechanisms exist to regulate GS activity in addition to phosphorylation/dephosphorylation. For example, binding of G-6-P to GS unfolds the enzyme resulting in allosteric activation and causes conformational changes in GS that favour dephosphorylation of the enzyme. Furthermore, translocation of GS to glycogen particles in response to stimuli (i.e. insulin) is an additional mechanism whereby GS activity is regulated (Greenberg et al., 2006).

During exercise muscles rely on glycolytic pathways to provide ATP for continued contraction. Glycogen breakdown is controlled by the enzyme glycogen phosphorylase, which in turn is activated by a number of stimuli related to muscle contraction (e.g. calcium) thereby allowing glycogen breakdown to change in parallel with the energy demands during exercise.

Repletion of glycogen stores involves an increase in GS activity after exercise. However, despite net glycogen breakdown during exercise, mechanisms exist to activate GS and therefore increase glycogen synthesis during exercise in an insulin-independent manner. An increase in GS activity in exercise appears to be dependent on the mode, duration and intensity of the exercise as some studies have demonstrated a reduction in GS activity during high intensity exercise. The insulin independence of GS activation during exercise suggests that exercise and insulin utilise different signalling pathways to activate GS. These mechanisms may be related to glycogen levels themselves, phosphatase (PP1) and kinase (GSK3, PKA, AMPK) activation and allosteric factors (G-6-P) (Nielsen and Richter, 2003).

1.2.6 Carbohydrate oxidation – oxidative glucose disposal

The PDH enzyme is part of the multi-enzyme pyruvate dehydrogenase complex (PDC), which catalyses the physiologically irreversible decarboxylation of pyruvate to acetyl CoA and is often referred to as a “gatekeeper” in the oxidation of carbohydrate. Therefore PDC links the degradation of intracellular glycogen and extracellular glucose via glycolysis, as well as the oxidation of extracellular pyruvate and lactate, to the energy requirements of the cell (Holness and Sugden, 2003). Moreover, when the glucose supply is high, the combination of acetyl CoA with oxaloacetate provides a precursor for malonyl-CoA production. Malonyl-CoA can limit the mitochondrial uptake (and therefore oxidation) of FFA via inhibition of carnitine palmitoyltransferase I (CPT1). On the other hand, when glucose availability is low or FFA supply and oxidation is sufficient to meet the cellular energy demands, PDC activity is suppressed, limiting the conversion of pyruvate to acetyl-CoA. This response to glucose scarcity may be crucial for glucose conservation (Holness and Sugden, 2003). Flux through the PDC is tightly regulated to maintain glucose homeostasis during both the fed and fasting states. This regulation is achieved via a combination of three major mechanisms: 1) reversible phosphorylation/dephosphorylation, 2) modifications of the activities of the regulatory components by the redox state and acetyl CoA/CoA ratio and 3) transcriptional regulation of the regulatory components (Patel and Korotchkina, 2006).

The PDC complex contains two specific regulatory enzymes, pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP). These two enzymes catalyse a phosphorylation/dephosphorylation cycle involving specific serine residues on the PDH enzyme (Sugden and Holness, 2003). The phosphorylation of the PDH completely inactivates the PDC and therefore the activity of PDC reflects the balance between the activities of PDK (which phosphorylates and inactivates PDH) and PDP (which dephosphorylates and activates PDH) (Linn et al., 1969). The regulation of these enzymes is also tightly regulated. The PDP enzymes (PDP1 and PDP2) are variably expressed in tissues but the dominant PDP1 enzyme in skeletal muscle requires magnesium and is stimulated by calcium (Huang et al., 1998). Short term regulation of PDK includes its inhibition by pyruvate and its activation by acetyl CoA and NADH – products of the PDC reaction and FFA oxidation (Kerbey et al., 1976). To date, four isoforms of PDK have been identified (PDK1-4) and each exhibits tissue specific regulation. PDK1 is found in the heart, pancreas and skeletal muscle; PDK2 is ubiquitously expressed in the fed state; PDK3 has a limited tissue distribution and PDK4 is expressed at high levels in the heart, skeletal muscle, kidney, liver and pancreas (Bowker-Kinley et al., 1998). The relative catalytic activity of the PDK isoforms towards PDH varies such that PDK2 and PDK4 exhibit the highest activity (Bowker-Kinley et al., 1998). In addition, each isoform is differentially affected by short term regulatory metabolites. For example, PDK2 is most sensitive to inhibition to

pyruvate where as PDK4 is relatively insensitive to pyruvate but instead is more sensitive to an increase NADH:NAD ratio (Bowker-Kinley et al., 1998).

1.3 Cellular mechanisms of insulin resistance

Insulin resistance, defined as a state of reduced responsiveness to normal circulating levels of insulin, plays a major role in the development of type 2 diabetes. Historically, insulin resistance has been viewed in terms of the effects of insulin on glucose metabolism. As such, early studies focused on the reduction in early insulin signalling events including those affecting the structure and number of insulin receptors itself to those affecting the phosphorylation of early effectors of the insulin signalling cascade. However, more recent analysis has revealed that insulin resistance also involves impairments in the enzymes that regulate glycogen synthesis and fat and carbohydrate metabolism, which lie downstream of insulin signalling pathways. In this section, a discussion of the cellular mechanisms involved in insulin resistance begins with an overview of the defects along the insulin signalling pathway and then progresses to consider mechanisms further downstream of these pathways, including glycogen synthesis and substrate oxidation, that may influence insulin resistance. At the end of this section (see 1.4), a summary is provided which attempts to collate all the currently available data and postulate an all-encompassing hypothesis for the mechanisms that may determine insulin resistance.

1.3.1 Insulin resistance and insulin signalling pathways

1.3.1.1 Insulin receptor number and affinity

Some early studies documented a small reduction in insulin binding in type 2 diabetic patients, due to a reduction in the number of insulin receptors (Freidenberg et al., 1987; Caro et al., 1986, 1987). In addition, defects in insulin receptor internalisation and processing in monocytes have been reported (Trischitta et al., 1989). These data are far from consistent however, and it should be noted that these defects in insulin receptor number and affinity have not always been reported in the two most important tissues for glucose disposal - muscle and liver (Klein et al., 1995). Sequencing of the insulin receptor gene has not revealed any physiologically significant mutations in type 2 diabetics, excluding the possibility of a structural gene abnormality in type 2 diabetic subjects (Moller et al., 1989). However, mutations of the insulin receptor gene have been reported in some rare genetic syndromes with severe insulin resistance (Moller et al., 1990; Hashiramoto et al., 2005; Taylor et al., 1992).

1.3.1.2 Insulin receptor phosphorylation

Most studies have found reduced tyrosine kinase activity of the insulin receptor in insulin resistance and type 2 diabetics, either in the form of

reduced substrate phosphorylation or autophosphorylation (Comi et al., 1987; Sesti et al., 2001; Haring et al., 1982; Nyomba et al., 1990; Nolan et al., 1994). This may be an acquired defect as insulin receptor activity can be improved following normalisation of fasting plasma glucose (for example by weight loss) (Freidenberg et al., 1988). In humans *in vivo*, studies employing the hyperinsulinaemic-euglycaemic clamp technique have demonstrated a large reduction in insulin receptor tyrosine phosphorylation and increased serine phosphorylation in type 2 diabetics (Dresner et al., 1999; Griffin et al., 1999). There are over 70 potential serine phosphorylation sites in IRS1 and, in general, this serine phosphorylation seems to negatively regulate insulin signalling suggesting that it may have a role in the pathogenesis of insulin resistance. Many IRS kinases including extracellular signal-regulated kinase (ERK) (Bouzakri et al., 2003), S6 kinase (Harrington et al., 2004) and c-jun-N-terminal kinase (JNK) (Miller et al., 1996) are activated by insulin suggesting that serine phosphorylation may be related to negative feedback of the insulin signalling pathway. In particular, serine phosphorylation of the insulin receptor in insulin resistance may be linked to fat metabolites and nPKC activation (see 1.3.1.4) (Savage et al., 2005). The activation of Nuclear Factor κ B (NF- κ B) mediated pathways also have been shown to inhibit insulin signalling through increased serine phosphorylation of IRS1 and may be a mechanisms that mediates cross talk between signalling pathways (Kim et al., 2001; Itani et al., 2002).

1.3.1.3 Glucose transport

Glucose transporter activity in type 2 diabetic patients has consistently been shown to be lower in both adipocytes (Kashiwagi et al., 1983) and muscle (Zierath et al., 1996; Krook et al., 2000). However, GLUT4 mRNA levels in muscle tissue extracted from type 2 diabetics are unchanged, or in some cases are increased when compared to control subjects (Pedersen et al., 1990; Kahn et al., 1991), whereas GLUT4 mRNA in adipose tissue is markedly reduced in type 2 diabetic humans and rodent models of diabetes (Kahn et al., 1991), suggesting that GLUT4 expression is subject to tissue specific regulation.

1.3.1.4 Novel PKCs and insulin resistance

A number of the novel PKC isoforms have been associated with the negative regulation of the insulin signalling pathway. This is in contrast to the positive effects of the α PKCs on GLUT4 translocation and glucose uptake (see 1.2.3). As mentioned above, these nPKCs include δ , ϵ , η and θ isoforms and require DAG but not calcium for their activation, and as such have consistently been linked to insulin resistance associated with increased lipid availability. Lipid infusion in rats and humans impairs insulin stimulated glucose disposal in muscle and is associated with concomitant activation of PKC- δ and θ (Itani et al., 2002; Yu et al., 2002; Dey et al., 2006; Shulman, 1999). It has been suggested that one mechanism whereby PKC- δ and θ reduce insulin

sensitivity is via serine phosphorylation of the IRS and this has recently been demonstrated *in vitro* (Greene et al., 2004). This serine phosphorylation may reduce the ability of IRS to activate PI3K and thus contribute to a reduction in insulin stimulated glucose uptake (see 1.2.2) and glycogen synthesis (see 1.2.5). An additional nPKC, PKC- ϵ , has recently been shown to be upregulated in the skeletal muscle of the diabetic *Psammomys obesus* rat (Ikeda et al., 2001).

1.3.2 Insulin resistance and glucose phosphorylation

HKII is regulated by insulin and, in response to physiological hyperinsulinaemia, HKII activity, protein and mRNA content are all increased in healthy non-diabetic subjects (Mandarino et al., 1995). It has been reported that in type 2 diabetes, glucose phosphorylation by HKII is reduced under basal conditions and in response to insulin infusion (Kruszynska et al., 1998). However, in the same study, basal and insulin mediated HKII activity was not reduced in insulin resistant obese subjects, suggesting that a reduction in HKII activity is probably secondary to the diabetic state (Kruszynska et al., 1998). This idea is consistent with animal models of induced diabetes in which HKII activity can be partly restored by insulin therapy (Braithwaite et al., 1995; Frank and Fromm, 1986).

1.3.3 Insulin resistance and glycogen metabolism

The importance of GSK3 to GS activation and insulin resistance is supported by several lines of evidence. Firstly, in poorly controlled type 2 diabetics, GSK3 protein expression and activity is upregulated (Nikoulina et al., 2000). However, in better controlled type 2 diabetics, it has recently been shown that GSK3 activity is normal despite insulin mediated activation of GS being almost absent (Hojlund et al., 2003), suggesting that increased GSK3 activity is more a feature of severe insulin resistance. In that study, insulin mediated dephosphorylation of GS at sites 3a + 3b was the same in normal and diabetic subjects but this dephosphorylation was counteracted in the diabetics by an insulin mediated phosphorylation of sites 2 + 2a on GS (Hojlund et al., 2003). Interestingly, alternative kinases associated with insulin resistance (e.g. PKC, 1.3.1.4) are able to phosphorylate GS on these sites (Ahmad et al., 1984).

More consistent evidence for a role of GSK3 in insulin resistance comes from studies employing GSK3 inhibitors. The administration of a number of chemical compounds, including lithium, malemides, aminopyridine derivatives and phosphopeptide pseudosubstrates, both acutely and chronically, has been shown to activate GS and lead to improvements in insulin action (reviewed in Cohen and Goedert, 2004). These studies suggest that GSK3 inhibitors may be a useful target in the treatment of type 2 diabetes.

In type 2 diabetics, exercise may improve blood glucose levels and overcome insulin resistance and this effect has been attributed, in part, to an increase in glycogen synthesis (Perseghin et al., 1996; Price et al., 1996) as a result of increased GS mRNA (Dela et al., 1995) and GS activity in the post exercise period (Christ-Roberts et al., 2003, Cusi et al., 2001). These studies suggest that therapies which mimic the effects of exercise on carbohydrate metabolism could prove to be potent therapeutic tools in the treatment of type 2 diabetes.

1.3.4 Insulin resistance and substrate oxidation

Carbohydrate and fat are the primary fuel sources for mitochondrial ATP production in human skeletal muscle. An interaction between the oxidation of these two fuels has historically been suggested as a potential mechanism underlying insulin resistance in skeletal muscle. Randle and colleagues proposed the concept of the “glucose-fatty acid cycle” in the 1960’s whilst studying rat heart and diaphragm muscle (Randle et al., 1963). The main features of this concept were that increased fat oxidation in muscle would inhibit both pyruvate dehydrogenase (PDH) and phosphofructokinase (PFK) by accumulation of acetyl CoA and citrate, respectively. Inhibition of these enzymes would lead to reduced flux through the glycolytic pathway and thus an increase in glucose 6-phosphate concentrations, inhibiting HK and resulting in reduced glucose uptake and oxidation.

Insulin resistance is associated with increased PDK activity and subsequent reductions in PDC flux. PDK activity is increased in several oxidative tissues *in vivo* in response to nutritional manipulations that increase lipid supply and oxidation. For example, PDK expression and /or activity increases with starvation (Wu et al., 1998; Spriet et al., 2004; Pilegaard et al., 2003), insulin resistance induced by high fat feeding (Peters et al., 2001) and streptozotocin induced diabetes (Wu et al., 1998) and this has originally been attributed to an increase in FFA. However, recent studies have shown that PDK4 expression is downregulated by insulin independently of FFA (Lee et al., 2004) and as the above states of insulin resistance are characterised by insulin deficiency, it is possible that the increase in PDK4 is a consequence of the inability of insulin to suppress PDK4 expression (Kim et al., 2006). Recent studies have implicated FOXO1 in the regulation of PDK4 by insulin (Kwon et al., 2004; Furuyama et al., 2003; Kim et al., 2006) (see 1.2.1.3). FOXO1 is expressed in insulin sensitive tissues including the liver and skeletal muscle and is phosphorylated by AKT/PKB through the insulin signalling pathway and then translocates from the nucleus to the cytosol resulting in reduced transcriptional activity. Therefore, a reduction in insulin signalling due to insulin resistance would activate FOXO1 and lead to an increase in PDK4 expression. These pathways may provide a possible link between the insulin signalling pathways and the control of carbohydrate and fat oxidation and thereby provide an integrative model of insulin resistance.

1.4 Summary – The metabolic basis of insulin resistance

From the discussions above, it is apparent that insulin resistance can be influenced by any one of many mechanisms, ranging from insulin receptor tyrosine phosphorylation to glycogen synthesis to fat and carbohydrate oxidation. The question that remains is which of the mechanisms described above are the most important and which of them underlie insulin resistance?

A hypothesis to explain insulin resistance in a way which integrated many of these pathways was suggested by Randle in the early 1960's (Randle et al., 1963). Initial attempts to confirm this model in humans demonstrated that increasing plasma FFA levels during a hyperinsulinaemic clamp (e.g. by lipid infusion) reduced carbohydrate oxidation, increased fat oxidation and inhibited insulin stimulated glucose uptake (e.g. Bonadonna et al., 1989). However, it was later shown in more detailed studies that although lipid infusion did increase fat oxidation and reduce carbohydrate oxidation *in vivo*, there was no change in citrate or G-6-P in muscle or in glucose disposal for at least 3 h (Boden et al., 1991). In other words, the long delay between the rise in fat oxidation and the fall in glucose disposal and the lack of an increase in citrate and G-6-P could not be explained by the traditional Randle hypothesis.

It was also shown that glucose oxidation was increased (not decreased as would be expected) in the leg muscle of type 2 diabetic subjects studied postabsorptively under conditions of fasting hyperglycaemia (Kelley and Mandarino, 1990). In retrospect, these findings perhaps were not surprising considering that in the skeletal muscle of healthy subjects, lipid, not carbohydrate, is the dominant oxidative fuel (Andres et al., 1956). Around the same time, it was shown that increased muscle glucose metabolism in skeletal muscle increased malonyl-CoA concentrations, which inhibited carnitine palmitoyl transferase (CPT)-1 and blocked FFA entry into the mitochondria (Winder et al., 1990). Reduced entry of FFA into the mitochondria might lead to an accumulation of activated FFA, or long-chain acyl-CoA concentrations, in the cell. An increase in the concentrations of fatty acyl CoAs can lead to increased DAG concentrations and, as outlined above, this can lead to nPKC activation. This PKC activation could lead to an increased serine phosphorylation of IRS1 and a reduction in PI3K and AKT/PKB activation leading, eventually, to a reduction in GLUT4 trafficking, reduced glucose uptake and reduced glycogen synthesis. Under this hypothesis, it appears that the rate-limiting step governing insulin resistance is indeed GLUT4 mediated glucose uptake.

Therefore, there is now increasing evidence to suggest that it is not increasing fat oxidation that produces insulin resistance, but instead that abnormalities in insulin action may arise as a result of an accumulation of various lipid species in skeletal muscle. In other words,

insulin resistance may begin with an accumulation of fatty acid metabolites and these metabolites may negatively regulate the insulin signaling pathway via mechanisms linked to DAG mediated PKC activation for example. Glucose oxidation could also contribute to insulin resistance at the same time via FOXO1 mediated PDK4 upregulation, leading to inhibition of PDH. Under this mechanism it is apparent that a reduction in glucose oxidation would be secondary to reduced insulin signalling. It is also possible to envisage the opposite; that an increase in lipid supply could lead to an increase in β -oxidation and increased acetyl CoA and NADH concentrations, leading to increased PDK4 activity, reduced carbohydrate oxidation and insulin resistance, although this idea is more consistent with the original Randle hypothesis. In summary, whilst the exact mechanisms that underlie insulin resistance are still under intense debate, current thinking points to an integration of the pathways that govern carbohydrate and lipid oxidation with those regulating insulin signaling. Clearly, a detailed understanding of the metabolic basis of insulin resistance will be crucial for the identification of potential drug targets and in the treatment of type 2 diabetes.

1.5 The search for type 2 diabetes candidate genes

From the discussions above (see 1.2 – 1.4), it is clear that defects in one or many proteins involved in skeletal muscle glucose uptake, glycogen synthesis or substrate oxidation may lead to severe defects in glucose homeostasis. Evidence that genes play an important role in the development of type 2 diabetes originally came from studies performed on twins. Studies in the USA and Denmark showed a high concordance rate (i.e. the presence of a given trait in both members of a pair of twins) for type 2 diabetes among monozygotic twins, reaching 41% and 55%, respectively (Newman et al., 1987; Harvald and Hauge, 1963). In dizygotic twins, the values were significantly lower (10% and 15%, respectively). Moreover, it is well known that some populations in the world have extremely high prevalence of type 2 diabetes, which cannot be attributed to environmental factors alone. An excellent example of this is the Pima Indian population; by the 1990s about 8% of young men and women aged 20-24 had the disease, and 3% of those aged 15-19 years were also affected in this population (Bennett, 1999; see also Knowler et al., 1978).

1.5.1 Simple and complex forms of type 2 diabetes

Based on the role of genetic factors, type 2 diabetes may be divided into two broad groups – those that are monogenic and those that are polygenic in nature. Monogenic forms, constituting only a small

proportion of type 2 diabetes cases ($\leq 10\%$), are simpler in nature and are frequently characterised by rare mutations in a single gene that have significant detrimental effects on the protein function. Monogenic forms are characterised by early age of diagnosis and usually a more severe clinical picture with the known forms commonly occurring as a result of a defect in insulin secretion and/or severe insulin resistance. Genetic background is far more important with the environment only slightly modifying the clinical outcome (Malecki, 2005, Malecki and Klupa, 2005).

Polygenic type 2 diabetes occurs far more frequently and accounts for ~ 90% of all cases. Polygenic forms of type 2 diabetes are thought to be the result of an interaction between environmental and genetic factors, which may actually constitute several genes. The variations associated with polygenic disorders are usually very common and often result in amino acid substitutions that modify a particular protein. Importantly, the causal alleles (alternative forms of a genetic locus) of the polymorphisms are often present in both healthy and type 2 diabetic patients with different frequencies and therefore the genes associated with polygenic diabetes can be considered “susceptibility loci” (Malecki, 2005, Malecki and Klupa, 2005).

1.5.2 Strategies used in the search for type 2 diabetes genes

Generally the strategies used to search for diabetes genes can be divided into two groups: (1) the genome scan and (2) the candidate gene approach. Whilst it is beyond the scope of this thesis to describe in detail the processes involved in each method, a brief outline of each will be provided as it has very significant relevance when discussing calpain-10.

1.5.2.1 The genome scan

By far the more complicated and laborious of the two techniques is the genome scan. This method is based on searching, using specific laboratory and analytical techniques, for a statistical signal that indicates the probability of co-segregation of a disease with a specific chromosomal locus. Any presence of a signal is then defined as a linkage between a trait under investigation and a chromosomal location (i.e. there is an association of that given disease with a specific area within a chromosome). The challenge is then to find which gene and, specifically, which mutations within that gene are responsible – regardless of its proposed biological role. This step is called positional cloning was the method used to identify calpain-10 as a type 2 diabetes candidate gene (see **1.5** and **1.6**).

1.5.2.2 The candidate gene approach

This method is much simpler than the genome scan and essentially involves selecting a gene based on its biological function (e.g., for a type 2 diabetes gene, one may look at genes known to be important for insulin action and/or insulin release) and then looking for mutations within its sequence using direct sequencing methodology. These approaches have been successfully applied to simple monogenic forms of type 2 diabetes (e.g., MODY3 gene and NEUROD1 gene for the genome scan and candidate gene approach, respectively).

For the study of polygenic diseases, investigators need to search for common variants that are present in a diseased group and a healthy control group, and look for the susceptibility alleles that are more prevalent in the diseased group. Importantly, in polygenic forms of type 2 diabetes, individual genetic susceptibility variants may have very minimal consequences at the individual level (e.g. small increases on individual risk for the disease), however for the whole population, the effects can be profound.

1.6 Positional cloning of type 2 diabetes genes

The number of chromosomal locations that harbour type 2 diabetes genes has been investigated using genome scans in hundreds of families of various ethnic and racial groups. The first of these studies conducted a genome scan on 170 Mexican-American families and found an association near 2q37 (Hanis et al., 1996). This susceptibility locus was termed NIDDM1 and was narrowed down to a target region of approximately 7 cM. Its effect was later to be shown to depend on the genotype at an unlinked locus and on its interaction with an area encompassing the CYP19 gene on chromosome 15 (Cox et al., 1999).

1.7 Calpain-10 as a type 2 diabetes gene

Continuing the work on the NIDDM1 locus described above, Horikawa et al. (2000) constructed a genomic contig (a group of clones representing overlapping regions of the genome) encompassing most of the 7 cM region that was most likely to include a type 2 diabetes gene. They identified 214 polymorphic sites across this region and tested for their association with type 2 diabetes. Five single nucleotide polymorphisms (SNPs) showed a difference in allelic frequencies between cases and controls and sequencing this region revealed two genes: calpain-10 (*CAPN10*) and G protein coupled receptor 35 (*GPR35*). None of the SNPs that were in the coding region of *CAPN10* or *GPR35* were associated with the disease. However, one of the

polymorphisms, designated SNP-43, a common G->A transition within intron 3 of CAPN10, was associated with the evidence for linkage in the NIDDM1 region in Mexican-American sib-pairs concordant with the at-risk genotype (G/G). Importantly, an increase in the frequency of the G allele was observed in patients when compared to controls. However, type 2 diabetes was only associated with a particular haplotype combination of two SNPs, SNP-43 and SNP-63 and the number of times a 32 nucleotide sequence (Indel-19) was repeated within the calpain-10 gene (Horikawa et al., 2000).

1.7.1 Replication of the association of calpain-10 with diabetes

For many genetic association studies, the importance of replication of the original findings cannot be overemphasised. For calpain-10 and diabetes in particular, replication studies have produced very contrasting results. Firstly, a number studies have confirmed the initial finding. In middle aged participants of the Atherosclerosis Risk in Communities study (269 prevalent diabetes cases and 1,159 non-diabetic control subjects), those with the G/G genotype at SNP-43 were more likely to have diabetes than those with either the G/A or A/A genotype (Garrant et al., 2002). Over the nine year follow up however, 166 of the control subjects developed incident diabetes, but the incidence of diabetes for those with the G/G genotype did not differ significantly from those with at least one copy of the A allele. The authors estimate that because of the high frequency of the G allele,

approximately 25% of the susceptibility of type 2 diabetes in African Americans may be associated with the G/G genotype at SNP-43. In a Mexican population, another SNP, SNP-44 was associated with type 2 diabetes, but the haplotype combination was not (del Bosque-Plata et al., 2004). Malecki et al. (2002) genotyped 229 type 2 diabetic and 148 control Polish subjects at SNP-43, -19 and -63 and found an association with a novel haplotype combination but not with the previously identified haplotype or the SNPs individually. Similarly in the Korean population, a novel diplotype in the calpain-10 gene was associated with type 2 diabetes (Kang et al., 2006). In the British population, Lynn et al. (2002) demonstrated that subjects with the G/G genotype at SNP-43 had higher 2 h plasma glucose levels during an oral glucose tolerance test (OGTT) compared to those with the G/A and A/A genotypes. Similarly those with previously described risk haplotype had higher fasting and 2 h plasma glucose compared to the rest of the study population (Lynn et al., 2002).

Additional studies have also investigated the association of calpain-10 SNPs and type 2 diabetes related physiological traits. For example, the A allele at SNP-43 was associated with intra-abdominal fat area and high insulin levels during an OGTT (Pihlajamaki et al., 2006). Comparing 395 diabetic and 298 control subjects, Orho-Merlander et al. (2003) not only showed an association with calpain-10 SNPs and type 2 diabetes but also suggested an association between variation in the calpain-10 gene and elevated plasma FFA and serum insulin

concentrations. In common with these findings, Carlsson et al. (2004) found that carriers of the G allele at SNP-43 had significantly elevated triglyceride levels compared to carriers of the G/A alleles, whereas Goodarzi et al. (2005) provided evidence for a role of calpain-10 variants in atherosclerosis in a population enriched for both atherosclerosis and insulin resistance. They also showed that calpain-10 affected insulin secretion and insulin sensitivity (Goodarzi et al., 2005). Other studies have also investigated the association between calpain-10 variation and insulin mediated glucose disposal and insulin secretion. In a comprehensive analysis Tripathy et al. (2004) showed that subjects with the SNP-44 (T/T) and SNP-43 (G/G) genotypes had lower levels of insulin stimulated glucose disposal.

However, a large number of studies that have investigated a link between calpain-10 variants and type 2 diabetes have not found one and this is particularly the case in many European populations. In a study of 1,603 Finnish subjects, Fingerlin et al. (2002) found no association between SNP-43, -19 and -63 and an increased risk of type 2 diabetes. In a large number (n = 6018) of Danish whites, Jensen et al. (2006) recently found no association between SNP-43 or -44 and type 2 diabetes. Nor was there any association between these SNPs and type 2 diabetes related traits such as obesity (Jensen et al., 2006). Daimon et al. (2002) reached a similar conclusion when they studied a Japanese cohort of 81 diabetic subjects and 81 non-diabetics. Similarly, in a Scandinavian population, Rasmussen et al. (2002) failed to find an

association with the three locus haplotype combination and type 2 diabetes or related traits (insulin resistance and insulin release). In a British group of subjects, Evans et al. (2001) did not find any association of type 2 diabetes with calpain-10 variants, either individually or as part of the haplotype combination. However, in keeping with previous studies (del Bosque-Plata et al., 2004) there was an association with the rare SNP-44 C allele in the British population (Evans et al., 2001). A variety of other studies have similarly found no association between calpain-10 variants and type 2 diabetes (Baier et al., 2000; Hegele et al., 2001; Tsai et al., 2001; Sun et al., 2002; Horikawa et al., 2003; Iwasaki et al., 2005; Einarsdottir et al., 2006).

1.7.2 Meta-analyses and the association of calpain-10 and diabetes

As a result of the inconsistencies surrounding the confirmation of an association between calpain-10 variants and type 2 diabetes, there have been a number of meta analyses published in an attempt to clarify the association between calpain-10 and diabetes. The first meta-analysis focused on the association between the SNP-44 genotype and type 2 diabetes (Weedon et al., 2003). The authors identified 10 case/control studies consisting of 3,303 subjects spread across a number of ethnic groups. The frequency of the rare risk C allele at SNP-44 varied from 6% in Mexican Americans to 25% in Botnians (a population from the Botnia region in Western Finland). Combining the data from these studies together with a new study performed as part of

the meta analysis confirmed the association of SNP-44 with type 2 diabetes (Weedon et al., 2003). Interestingly, SNP-44 is in perfect linkage disequilibrium with the missense mutation Thr504Ala and two additional SNPs in the 5'-UTR of calpain-10 and therefore may not be the causal variant but rather a marker for it (Weedon et al., 2003).

A more comprehensive meta-analysis combined 21 published studies, one third of which involved white Europeans, one third Americans (including Pima and Mexican Americans) and the remaining third involved Asians (Song et al., 2004). This study analysed the effect of all of the major calpain-10 SNPs for an association with type 2 diabetes. There were significant differences in the allele frequencies of SNP-43, -44, Indel-19 and SNP-63. Most notably there was a large variation in the frequency of the risk G allele at SNP-43, which varied from 0.62 in Pima Indians to 0.96 in Japanese. However, the allele frequencies were not different between cases and controls. Analysis of the population based studies using a recessive model revealed that individuals homozygous for the G allele at SNP-43 had a 19% increased risk for type 2 diabetes, but using a dominant effect model there was no association. In the family based studies (e.g. Evans et al., 2001) the authors found no evidence for overtransmission of the G alleles at SNP-43 from the heterozygous parents to diabetic offspring. There were also considerable differences in the frequencies of haplotype combinations across different populations. When investigating calpain-10 variants and diabetes related quantitative traits,

individuals homozygous for the G allele at SNP-43 had higher systolic blood pressure, but this was based on data from only three trials. Other metabolic traits, including fasting and 2 h blood glucose and serum insulin concentrations (during an OGTT), were not different between carriers of the G/G genotype and carriers of the A allele. The authors calculated that to detect the modest association between calpain-10 and type 2 diabetes using a population based approach, studies required 2,188 cases and an equal number of controls, which none of the studies to date has used. A similar problem was found to exist amongst family based studies.

The most recent meta-analysis attempted to resolve the inconsistent association between calpain-10 variants and type 2 diabetes in Europeans (Tsuchiya et al., 2006). For this analysis, all case/control studies that genotyped individuals at SNP-43, Indel-19 and SNP-63 were considered in addition to an additional study of 2,514 subjects performed for the purpose of the meta-analysis. The final study group consisted of 3,237 cases and 2,935 controls (therefore matching the above criteria). The results from the meta-analysis indicated a trend towards a significant association of the SNP-43 G allele with type 2 diabetes ($P=0.09$). Analysis of the haplotype combinations indicated that there was some limited evidence for an association with three separate haplotypes.

In summary therefore, these meta-analyses indicate that variants in the calpain-10 gene are associated with type 2 diabetes, although it is clear from numerous studies that the impact of these variants is considerably variable across populations. Nevertheless, the finding of an association between calpain-10 and type 2 diabetes was remarkable for a number of reasons, but not least because calpain-10 was an unknown gene with no previous links to disease, including type 2 diabetes. Moreover, whilst the calpain superfamily was well known and well studied (1.7), it was difficult to hypothesise exactly how calpain-10 might effect insulin action and/or insulin release based on what was known about the actions and the regulation of the calpain system.

1.8 The calpain superfamily

Calpains are a family of intracellular calcium activated cysteine proteases that are involved in the partial proteolysis of specific protein substrates. The first documentation of calpain in mammalian cells was reported 40 years ago (Guroff et al., 1964) and calpain was first purified from porcine skeletal muscle (Dayton et al., 1976a; Dayton et al., 1976b) and has since been found in most organisms studied ranging from insects to humans. Most tissues that are known to contain calpain also contain a specific inhibitor of their activity known as calpastatin, which was also purified from porcine skeletal muscle shortly after the first documentation of calpain in the same tissue (e.g. Murachi et al., 1980).

The calpain family currently consists of 14 members (Fig 1.1), but by far the most studied of these is the so-called ubiquitous calpains, μ - and m-calpain (or calpain 1 and 2). The nomenclature of the two ubiquitous calpains is governed by their calcium requirements for half-maximal activity – micromolar (3-50 μ M) and millimolar (0.4-0.8 mM) calcium concentrations for μ - and m-calpain respectively (Cong et al., 1989).

1.8.1 Properties of μ - and m-calpain – the ubiquitous calpains

cDNAs encoding μ - and m-calpain have been cloned and sequenced for human (Ohno et al., 1986), monkey, mouse, rat (DeLuca et al., 1993), bovine, porcine (Smith et al., 2001), rabbit (Emori et al., 1986a; Emori et al., 1986b) and chicken (Ohno et al., 1984) calpains, and much is known about the structure of these two proteins as a result. A brief discussion of the structure of these ubiquitous calpains is essential when considering the domain structure and possible actions of calpain-10.

1.8.1.1 Domain structure

In their native form, the ubiquitous calpains exist as heterodimers with a common small regulatory subunit of 28 kDa, and a large 80 kDa catalytic subunit (Fig 1.2) (Aoki et al., 1986). Based on their amino acid structure, the ubiquitous calpains have been divided into six domains; domains I-IV form the larger 80 kDa subunit and domains V-VI constitute the smaller 28 kDa subunit (Fig 1.1 and 1.2). Recent crystallographic studies of rat (Hosfield et al., 1999) and human (Strobl et al., 2000) m-calpain, however, have shown that the 80 kDa subunit consists of six 'domains' – including a linker domain between domains III and IV and an 18 amino acid NH₂ terminal domain (Fig 1.1 and 1.2).

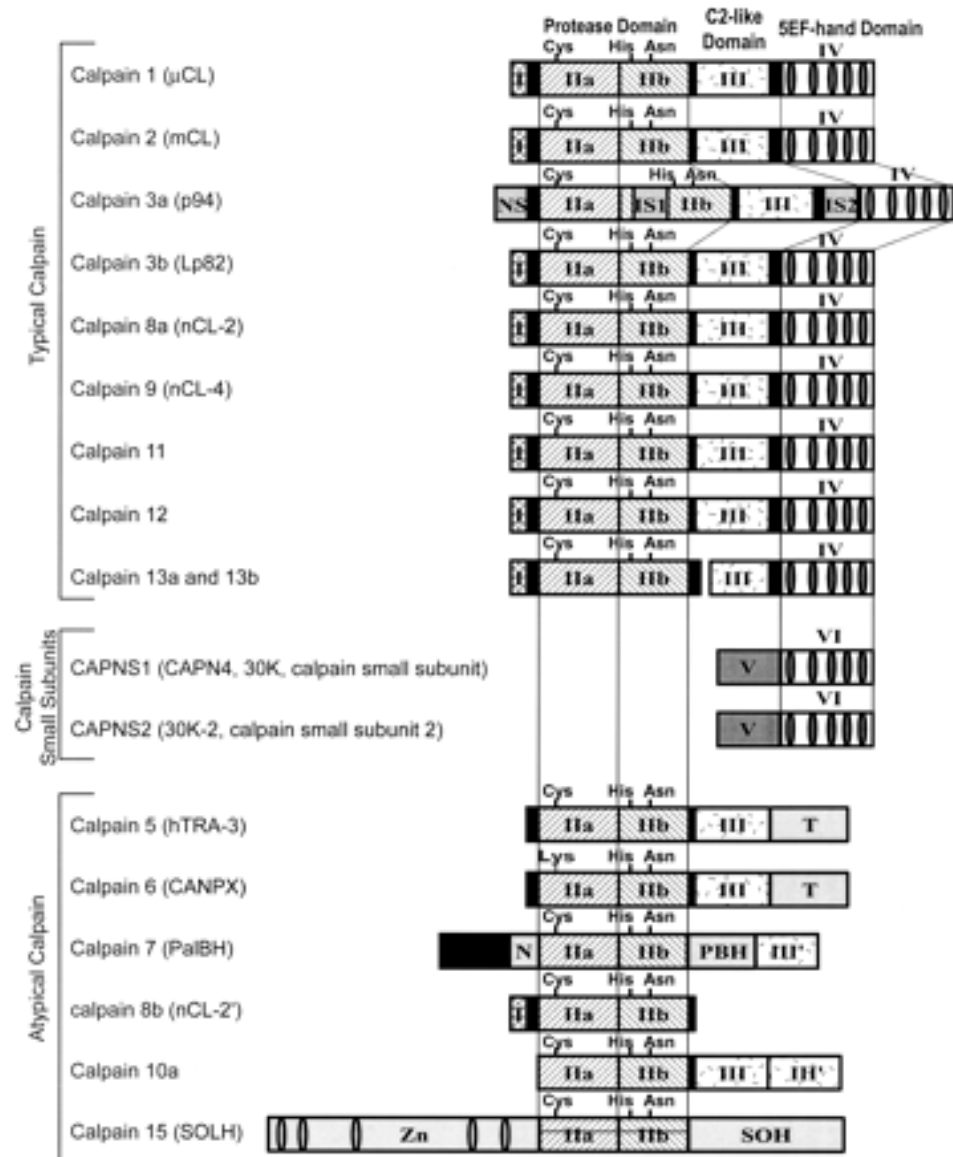


Figure 1.1 The domain structure of the calpain superfamily

The domain structure of both the ubiquitous calpains (μ - and m-calpain) is shown in addition to that of the atypical calpains (e.g. calpain-10). Generally, calpains are divided into as many as six domains (I – VI), each with distinct functions. The “T” domain has been found in the atypical calpains and replaces the calcium binding domain IV. Adapted from Suzuki et al. (2004).

1.8.1.2 Large 80 kDa subunit

The catalytic domain II of the 80 kDa subunit is made up of an amino acid triad consisting of a Cys residue at position 115 (μ -calpain) or 105 (m-calpain), a His residue at position 272 (μ -calpain) or 262 (m-calpain) and an Asn residue at position 296 (μ -calpain) or 286 (m-calpain) (Goll et al., 2003). This catalytic triad is characteristic of other cysteine proteases such as papains or cathepsins, although the remainder of domain II shares little sequence homology with these other proteases (Goll et al., 2003). Domain II is, however, highly conserved amongst species (Goll et al., 2003). The crystallographic studies of m-calpain have shown that domain II consists of two sub-domains, domains IIa and IIb (Fig 1.1 and 1.2) (Moldeveanu et al., 2002). Importantly for studies of calpain activation, the active site Cys is in domain IIa, whereas the His and Asn residues are in domain IIb.

Domain III has yet to be assigned a definitive function, although it is commonly referred to as an 'electrostatic switch' (Ma et al., 2001), whereby it may play a role in the activation of calpain through a number of interactions with the other calpain domains, most notably with the catalytic domain II. The C2 like fold revealed by crystallographic studies of m-calpain suggest a role for domain III in membrane association processes (Hosfield et al., 1999), which may also be an important step in calpain activation. Domain III also contains a putative

EF-hand calcium binding site at the boundary with domain II (Andressen et al., 1991).

Domain IV displays some homology to calmodulin and, based on the amino acid structure, contains four sets of sequences that predict EF-hand calcium binding sites (Goll et al., 2003). The crystal structure of m-calpain shows that domain IV may actually begin at amino acid residue 530, and include the EF-hand sequence at 541-552 (Fig 1.2) (Strobl et al., 2000). This EF-hand sequence may be important in the dimerisation of the 28 and 80 kDa subunit (Goll et al., 2003).

1.8.1.3 Small 28 kDa subunit

Domain V contains a Gly rich region at the NH₂ terminus, and is therefore thought to be to a hydrophobic domain that may bind phospholipid (Imajoh et al., 1986). Domain VI, like domain IV of the larger subunit is often referred to as a calmodulin-like domain, and amino acid structure predicts four EF-hand calcium binding sites (Ohno et al., 1986). Like domain IV however, the crystal structure has revealed an additional calcium binding site at positions 108-119 (Fig 1.2) (Blanchard et al., 1997; Lin et al., 1997).

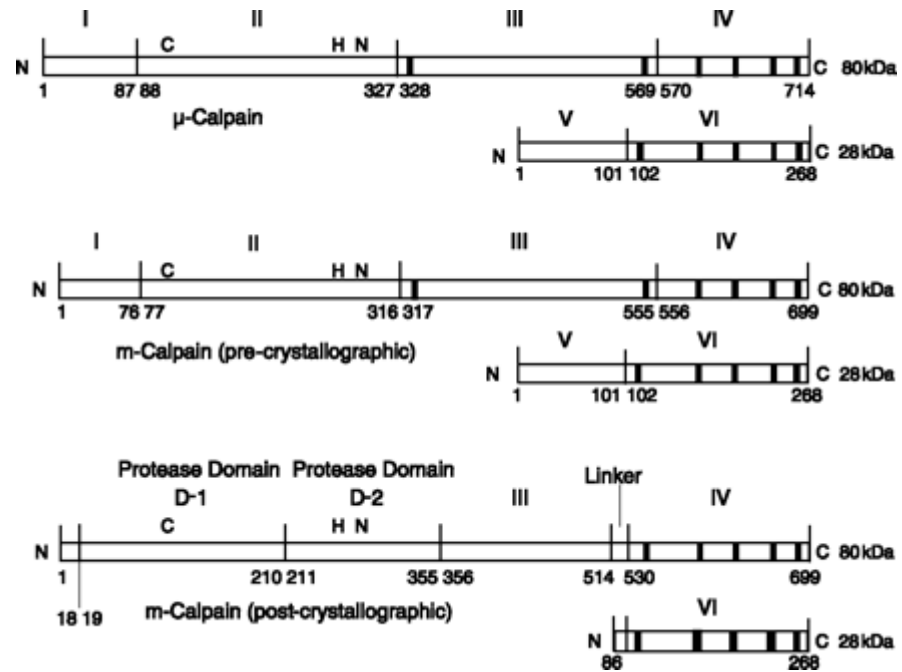


Figure 1.2 Domain structure of μ - and m-calpain.

Shown is the domain structure of μ - and m-calpain and also the structure of m-calpain after studies of its crystallographic structure, as outlined in the text. The solid black bars in domain IV represent EF hand calcium binding sites. Adapted from Goll et al. (2003).

1.8.1.4 Activation of calpain

The exact mechanisms of calpain activation are currently not well understood, although significant advances are now being made following the publication of the crystal structure of m-calpain. It has been established that calcium is essential for μ - and m-calpain activity, within specific ranges (3-50 μ M and 400-800 μ M for μ - and m-calpain respectively) (Goll *et al.*, 2003). However, free calcium concentrations inside cells (0.3 – 1.2 μ M) are considerably lower than those needed by both calpains, and some mechanism must therefore exist to lower the calcium requirements of the calpains. A number of possible mechanisms have been suggested, including autolysis (Suzuki *et al.*, 1981; Dayton *et al.*, 1982), membrane association (Cong *et al.*, 1989) and the involvement of so-called activator proteins (Pontremoli *et al.*, 1987a; Pontremoli *et al.*, 1987b).

1.8.1.5 Regulation of calpain activity by calpastatin

Whilst it is beyond the scope of this thesis to discuss in full the proposed mechanisms that regulate calpain activity, one aspect of calpain regulation i.e. calpastatin inhibition, requires consideration. During the initial purification of m-calpain, it was discovered that muscle extracts with calpain activity also contained an inhibitor of this activity. The name calpastatin was proposed for this inhibitor by Takashi Murachi in 1979 (see Murachi *et al.*, 1980). Historically, it has been

difficult to characterise calpastatin for a number of reasons, most notably because it is highly susceptible to proteolytic degradation and because a number of isoforms exist from a single gene. At least 8 different isoforms have been identified in tissues thus far, and frequently more than one isoform exists in a single tissue (e.g. Geesink *et al.*, 1998). The use of at least four alternative promoters and the ability to alter domain L by alternative splicing means that it is possible to produce a number of calpastatin polypeptides from a single gene (e.g. Takano *et al.*, 2000; Parr *et al.*, 2004). The physiological significance of the various isoforms remains unknown. Calpastatin is the only known inhibitor specific for the calpains, and does not inhibit any other proteases for which it has been tested (e.g. papain, cathepsin) (Goll *et al.*, 2003). Exactly how calpastatin regulates calpain activity *in vivo*, however, is not fully understood. What is known is that calcium is required for calpastatin to bind to and inhibit the calpains (Kapprell and Goll, 1989), that calpastatin inhibition is reversible and calpastatin can be released from calpain by chelating calcium with EDTA. In most cases, the concentration of calcium required for binding of calpastatin to calpain is much lower than that required to initiate proteolytic activity of the calpains (Kapprell and Goll, 1989), although it is much higher than free calcium concentration inside cells. There is currently no evidence that calpastatin actually binds calcium itself.

Calpastatin has been shown to bind to both domains IV and VI of the calpain molecule (Nishimura and Goll, 1991), and it might be the case that by doing so calpastatin is poised to prevent any calpain activity in the absence of calcium (Melloni et al., 2006). This is supported by studies showing that calpastatin and calpain colocalise inside cells (Kumamoto et al., 1992). Because of the differences in the calcium concentrations required for calpastatin binding and calpain activity, under certain circumstances calpains must be active when calpastatin is bound to calpain.

Importantly for the study of calpain-10, it appears that calpastatin does not inhibit the atypical, homodimeric forms of calpains as it must bind to three sites on the calpain molecule to be effective, and this includes calcium dependent binding to domain VI in the small 28 kDa subunit. For example, calpastatin does not inhibit the weak proteolytic activity of expressed domains IIa/IIb (Moldovenveanu et al., 2002) and only slightly inhibits the activity of lens specific calpain-3 (LP82), which does not possess a small subunit (Nakamura et al., 1999). Therefore in these and perhaps other atypical calpains (e.g. calpain-10), one of the three critical calpastatin binding domains is absent.

1.8.2 Physiological functions of calpain

The ubiquitous calpains cleave polypeptides at a limited number of sites to leave large, mostly catalytically active fragments, and therefore, it is accurate to describe calpains as being regulatory enzymes. They do not appear to have a broad digestive function like the proteasome or the enzymes contained within the lysosome.

1.8.2.1 In vitro and in vivo substrates

A large number of proteins (>100) are known to be cleaved by calpain in *in vitro* assays. Generally, these proteins fall into one of four categories: 1) cytoskeletal proteins, 2) kinases and phosphatases, 3) membrane associated proteins and 4) transcription factors. Many of the cytoskeletal proteins involved in linking the cytoskeleton to the plasma membrane are cleaved rapidly by the calpains and include talin, vinculin, spectrin, filamin, band 4.1a, band 4.1b and ezrin. Similarly, most of the intermediate filament proteins such as desmin and vimentin are cleaved rapidly by the calpains. Calpain cleavage of the cytoskeletal proteins severs their cross-linking abilities in most cases. Identification of *in vivo* substrates of calpain, however, has been difficult due to the non-specific nature of synthetic calpain inhibitors and in most cases it remains to be seen whether the same specific substrates exist under *in vivo* conditions.

1.8.2.2 Calpain and cytoskeletal remodelling

The proteolytic activity of the calpain system on skeletal muscle was originally identified by Busch et al. (1972) who established that isolated myofibrils treated with calpains exhibited rapid and extensive Z-disk degradation. Muscle tissue contains three classes of proteins: 1) the sarcoplasmic or cytoplasmic proteins that constitute ~ 30% of total muscle protein; 2) the myofibrillar or contractile proteins that make up ~ 55% of muscle protein; and 3) the stroma proteins (including the plasma membrane) that constitute the remaining ~ 10%.

Of the myofibrillar proteins, the calpains rapidly cleave titin (Suzuki et al., 1996; Taylor et al., 1995) and nebulin (Taylor et al., 1995) at sites near the Z-disk. In addition, the calpains cleave the intermediate filament protein desmin that attaches the Z-disk to the sarcolemma (Nelson and Traub, 1983). As a result, the proteins constituting the Z-disk, including α -actinin, are released and the Z-disk disappears, leaving a space in the myofibril. The released α -actinin can rebind to actin, indicating that it has been released from the Z-disk in a largely undegraded form (Goll et al., 1991). Close examination of muscle tissue in conditions of muscle wasting shows that the muscle often has abnormal Z-disks, and in some severe cases, the Z-disks are missing altogether (Goll et al., 2003). Moreover, the calpains also rapidly cleave troponins T (Goll et al., 2003) and I (Ho et al., 1994) and tropomyosin (Goll et al., 2003), and C-protein (Goll et al., 2003), which contribute to

stability of the thin and thick filaments, respectively. Thus the net result of calpains on skeletal muscle is the progressive disruption of the Z-disk leading eventually to its complete loss, the subsequent release of actin and myosin filaments and production of fragments of titin, nebulin, desmin and other proteins. Further degradation of these proteins likely involves the recruitment of other proteases (e.g. proteasome).

1.8.2.3 Calpains and skeletal muscle differentiation

One of the classical functions of the calpain system is the degradation of cytoskeletal/plasma membrane attachments. The proteins involved in cytoskeletal/plasma membrane interactions, such as talin (Muguruma et al., 1995), dystrophin (Yoshida et al., 1992) and spectrin (Yoshida et al., 1995) are rapidly cleaved in *in vitro* assays. Moreover, many studies have shown that calpains are located near the plasma membrane under certain conditions (Kumamoto et al., 1992).

A well studied example of cell fusion is fusion of skeletal muscle myoblasts during differentiation to form myotubes (Schollmeyer, 1986a, 1986b). During muscle development, myoblasts withdraw from the mitotic cycle, align, and then fuse to form multinucleated myotubes that accumulate skeletal muscle proteins such as actin, myosin and titin to form skeletal muscle fibres. The fusion of myoblasts involves extensive remodelling of the cytoskeletal/plasma membrane attachments. Kaur and Sanwai. (1981) first reported that calpain activity increased

significantly as L6 or L8 rat myoblast cell lines fused to form myotubes. Further evidence comes from studies that have injected the specific calpain inhibitor calpastatin directly into myoblasts just before fusion, which resulted in the complete abolition of fusion without killing the myoblast (Temm-Grove et al., 1999), and from studies employing antisense oligodeoxyribonucleotide strategies (e.g. Balcerzak et al., 1995). Specific inhibition of m-calpain synthesis by approximately 50% in rat myoblasts led to a corresponding reduction in myogenesis by approximately 70% (e.g. Balcerzak et al., 1995). Whilst the levels of calpain expression do not change during myoblast differentiation, the expression of calpastatin is significantly reduced prior to myoblast fusion and recovers following fusion (Barnoy et al., 1996). More recent experiments have shown that overexpression of calpastatin inhibits myoblast fusion and fusion-associated protein degradation, including talin and fodrin, which is also known as spectrin (Barnoy et al., 2005). It has also been reported that desmin, vimetin and fibronectin are also good substrates for calpains during myogenesis (Dourdin et al., 1999) and that the two calpains may have different roles in myogenesis (Cottin et al., 1994). Calpastatin overexpression also reduced the expression of myogenin which is one of the muscle specific transcription factors that determine the initiation and maintenance of the myogenic program (Barnoy et al., 2005).

Similarly, calpains have also been implicated in cell spreading and motility (Huttenlocher et al., 1997; Palecek et al., 1998). Early

experiments used cell permeable calpain inhibitors to show that integrin mediated cell migration was dependent on calpain activity (Huttenlocher et al., 1997). Reduced levels of μ -calpain in a Chinese hamster ovarian cell line also showed reduced migration rates with similar morphological changes (Huttenlocher et al., 1997). Inhibition of calpain activity via the overexpression of calpastatin was later found to inhibit the turnover of vinculin and zyxin-containing adhesive contact sites (Bhatt et al., 2002). Stable overexpression of calpastatin in C2C12 myoblasts similarly led to a complete abolition of multinucleated myotube formation confirming the critical role of calpains in skeletal muscle myogenesis (Dedieu et al., 2004).

1.8.2.4 Calpain and skeletal muscle growth (moved)

As mentioned above (see 1.8.2.1 and 1.8.2.2), calpains cleave a number of myofibrillar proteins and play an important role in cytoskeletal remodelling and myoblast differentiation *in vitro*. These findings led to the hypothesis that changes in skeletal muscle mass would be correlated with alterations in the expression of calpain and calpastatin. A number of lines of evidence now exist to support such a hypothesis.

Firstly, specific calpain inhibition by calpastatin overexpression in mice *in vivo* leads to marked skeletal muscle hypertrophy (Otani et al., 2004; Tidball and Spencer, 2002). Calpastatin overexpression has also been associated with elevated calcium calmodulin-dependent protein kinase

II (CaMKII) levels in muscle (Otani et al., 2004) and it is possible this may link calpain to skeletal muscle hypertrophy indirectly as CaMKII has been linked to the activation of additional proteolytic systems (Menconi et al., 2004).

Secondly, the administration of β -adrenergic agonists to animals results in large increases in the rate of accumulation of muscle mass and this treatment has been shown to affect the activity and expression of the calpain system. In particular, skeletal muscle calpastatin activity was significantly increased, the activity of μ -calpain was decreased and m-calpain was increased with the administration of β -agonists in skeletal muscle (Bardsley et al., 1992; Parr et al., 1992; Forsberg et al., 1989). These data suggest that reductions in μ -calpain activity may, in part, mediate the increase in muscle mass, which is consistent with the findings from calpastatin overexpressing mice. A bovine calpastatin promoter has been isolated and been shown to be functional in cell transfection studies and inducible by dibutyryl cAMP (Cong et al., 1998; Sensky et al., 2006) highlighting a possible mechanistic link between β -adrenergic stimuli and changes in the calpain system.

Jones et al. (2004) measured calpain expression following two weeks of limb immobilisation in humans. Following the immobilisation period there was an approximate 5% reduction in quadriceps lean skeletal muscle mass and the mRNA levels of the muscle specific calpain-3, but not μ - and m-calpain or calpastatin, were decreased with immobilisation

(Jones et al., 2004). Rehabilitation exercise following immobilisation led to an increase in muscle mass and an increase in the expression of μ - and m-calpain and calpastatin 24 h after the first bout of exercise. Calpastatin and m-calpain mRNA levels returned to basal levels after six weeks of rehabilitation, whilst μ -calpain continued to rise until at six weeks post rehabilitation they were higher than basal values. An early increase in μ -calpain with exercise is consistent with the β -agonist studies showing an initial increase in μ -calpain with an increase in muscle mass. These studies suggest that calpain may play a role in the initial remodelling of skeletal muscle that is necessary for hypertrophy. In addition a reduction in calpain-3 would be expected during atrophy as LGMD2A is due to a loss of calpain-3 substrate processing ability, not an increase. This may be related to a role of calpain-3 in the NF- κ B pathway, which has consistently been linked to the regulation of skeletal muscle mass (Hasselgren et al., 2005). Additionally, these studies indicate that of the ubiquitous calpain members, μ -calpain is the one most strongly associated most with skeletal muscle hypertrophy.

Finally, a possible role for calpain in skeletal muscle atrophy is further supported by the finding that calpain cleaves the transcription factor C/EBP β (see 1.8.2.5). Recent evidence suggests that this transcription factor is upregulated in states of skeletal muscle atrophy, including sepsis-induced muscle atrophy (Penner et al., 2002; Yang et al., 2005). Muscle atrophy during sepsis is associated with an increase in the DNA binding activity of C/EBP β in addition to an increase in the accumulation

of C/EBP β protein in the nucleus (Penner et al., 2002). Interestingly, C/EBP β binding sites are prevalent in the promoters of various genes of the ubiquitin-proteasome system (Penner et al., 2002) although specific evidence for a direct link between C/EBP β activation and an increase in proteasome expression and activity is currently not available. These data may provide a potential link between muscle atrophy and the calpain and ubiquitin-proteasome protease pathways.

In summary, therefore, numerous lines of evidence suggest that calpains participate in the disassembly of the Z-disk and therefore play an important role in skeletal muscle protein turnover. Clearly, the calpain system does work alone in this task and it is probably the case that the role of the calpain system is to target substrates for further degradation by other proteolytic systems. The ubiquitin-proteasome proteolytic pathway is essential for the development of muscle atrophy (Hasselgren et al., 2005) and it is likely that it acts alongside calpain activation, digesting released proteins into smaller peptide residues (Goll et al., 2003).

1.8.2.5 Calpain and signal transduction pathways

Calpains have been shown to cleave many of the kinases, phosphatases and cytoskeletal proteins involved in signal transduction pathways in *in vitro* assays, but it has been difficult to identify specific calpain cleavages *in vivo* that are specific to particular transduction

pathways. One of the first classical signalling proteins to be identified as a calpain substrate *in vitro* and one which is relevant to the development of insulin resistance was protein kinase C (PKC) (Kishimoto et al., 1983, 1989) (see 1.2.1.5 and 1.3.1.4). The authors used *in vitro* calpain cleavage assays to demonstrate that a number of purified PKC isoforms were cleaved by both μ - and m-calpain to produce a catalytically active fragment originally described as protein kinase M (PKM) (Kishimoto et al., 1983, 1989). Interestingly, this process of PKC cleavage was enhanced by the simultaneous presence of phospholipid and diacylglycerol. Part of the role of calpains in signal transduction may be related to their role in cytoskeletal/membrane interactions. Indeed, Liang et al. (2006) have recently linked the role of calpains in myogenesis to their cleavage of PKC and have shown, using μ - and m-calpain antisense oligonucleotides, that downregulation of μ - and m-calpain expression inhibited myoblast differentiation and altered the expression of various PKC isoforms including PKC α and PKC δ .

Recent data from transgenic mice overexpressing calpastatin highlighted a possible role for calpain in insulin signalling and GLUT4 translocation (Otani et al., 2004). It was shown that calpastatin overexpression does not affect glucose tolerance, but does lead to a reduction in AKT/PKB protein content and an upregulation in GLUT4 protein. Levels of GLUT4 mRNA were downregulated whilst other insulin signalling proteins were not affected (IR, IRS-1, PI3K). It is

suggested by the authors that the lack of an increase in glucose uptake that would be expected with the changes in GLUT4 protein can possibly be explained by the observed reduction in AKT/PKB. It is thought that the changes in GLUT4 were indeed a result of calpain inhibition because: 1) GLUT4 mRNA was reduced as was the expression of two transcription factors – MEF2A and MEF2D and 2) GLUT4 was shown to be cleaved by μ -calpain *in vitro* as part of their study.

In part two of these studies (Otani et al., 2006) it was reported that AMPK and CAMKII were increased 3-fold and it was hypothesised that these changes in the calpastatin overexpressing mice would lead to an increase in contraction mediated glucose uptake. AMPK is activated by increases in the AMP:ATP ratio and decreases in phosphocreatine, and thus is activated during muscle contractions, whereas CaMKII may be activated by calcium during muscle contraction. There is currently no evidence to support the idea that the calpain system is involved in the activation of AMPK or CaMKII. However, despite the increases in GLUT4, AMPK and CAMKII, there was no increase in contraction mediated glucose uptake when compared to wild type mice suggesting that inhibition of calpain results in impairment of a step in the GLUT4 translocation process downstream of the insulin- and contraction-signalling pathways. It is possible that calpain is involved in the very late stages of GLUT4 vesicle translocation and fusion and this has been suggested from studies of calpain-10 and glucose stimulated insulin release from the pancreas (see 1.9.2.4).

1.8.2.6 Calpain and transcription factors

The concept that calpains are involved in gene regulation comes largely from early studies showing that calpain cleaved transcription factors such as c-Jun (Hirai et al., 1991) c-Fos (Pariat et al., 2000), IkappaB and p53 (Gonen et al., 1997; Kubbatat and Vousdon, 1997; Pariat et al., 1997). Many of these studies have used non-specific calpain inhibitors and therefore the results have been questioned and it has been suggested that the proteasome and not the calpain system is responsible for the observed degradation of transcription factors (Goll et al., 2003). More recent studies, however, have used the more specific calpain inhibitor calpastatin to demonstrate a role for calpain in the cleavage of transcription factors. For example, calpastatin overexpression in L6 myoblasts led to significant increases in the protein levels of CCAAT/enhancer-binding protein β (C/EBP β) (Wei et al., 2006). Employing the use of mobility shift assays (EMSA) and luciferase reporter constructs, the authors showed that in myotubes treated with calpeptin the DNA binding activity and C/EBP β dependent gene activation was increased (Wei et al., 2006). More specific overexpression of μ - and m-calpain conversely reduced levels of C/EBP β in myoblasts expressing μ -calpain predominantly (Wei et al., 2006). Finally, co-immunoprecipitation revealed that calpains and C/EBP β interact at the protein level. Taken together, these results strongly suggest that calpain may play a role in the degradation and regulation of C/EBP β . These findings may be relevant in discussion of

the role of calpains in skeletal muscle atrophy given the proposed role of C/EBP in skeletal muscle wasting (Penner et al., 2002; Yang et al., 2005) (see 1.8.2.4).

1.9 Structure and function of calpain-10

1.9.1 Domain structure of calpain-10

An understanding of how calpain-10 differs structurally from the other calpain family members is critical for the understanding of calpain-10 function. Calpain-10 has been placed into the calpain family based entirely on sequence homology to the ubiquitous calpains. The gene encoding calpain-10 consists of 15 exons spanning approximately 31 kb (Fig 1.3). Analysis of human cDNA revealed a complex pattern of alternative splicing which leads to the generation of up to 8 different protein isoforms of approximately 672, 544, 517, 513, 444, 274, 139 and 138 amino acids (Fig 1.4) (Horikawa et al., 2000).

Calpain-10 is an atypical calpain in that domain IV has been replaced by a domain III like structure. Therefore it appears that its protease domain is followed by a tandem repeat of two domains that are similar to domain III. In the early calpain-10 literature (e.g. Horikawa et al., 2000), the last domain was originally described as a T domain, but it is now thought of as a III-like domain (Fig 1.4). In terms of domain structure, calpain-10 can be regarded as a close relative of other atypical calpain members, calpain-5 and -6 (Suzuki et al., 2004). Whilst the sequences around the active site triad (Cys-105, His-262 and Asn-286 for m-calpain and Cys-73, His-238, Asn-263 for calpain-10) are highly conserved among calpain family members, calpain-10 is different

from calpain-5 and -6 and other calpains in its amino acid sequence. Most importantly, residues in domain II that are thought important for calcium binding and which are common among the typical calpains are not conserved and, second, acidic and basic loops in domain III important for calcium and phospholipid binding are not present in the two domain III-like domains in calpain-10 (Suzuki et al., 2004). The result of this is that calpain-10 probably has none of the calcium binding sites normally found in the typical calpain family members (Suzuki et al., 2004). The regulation of calpain-10 activity by calcium, if indeed it is regulated by calcium, would therefore be expected to be different from the other typical calpain members (Suzuki et al., 2004). To date there has been no description of any protease activity of calpain-10 and detailed investigations into the structure and function of calpain-10 have been difficult due to problems of purifying the protein.

The mRNA of calpain-10 is expressed ubiquitously in all adult and fetal human tissues examined (Horikawa et al., 2000), and similar ubiquitous mRNA and protein expression has been confirmed in rats (Ma et al., 2001). The mRNA level of calpain-10 varies between tissues but appears to be highest in the heart, followed by the brain, liver, kidney, and pancreas in humans (Horikawa et al., 2000). Three of eight splice variants identified for calpain-10 completely lack an intact domain II and thus probably have no protease activity (Fig 1.4) (Horikawa et al., 2000). The expression of calpain-10a and -10f appear to represent the majority of calpain-10 transcript in skeletal muscle tissue (Yang et al.,

2001). Some calpain-10 is detectable in the soluble fraction by Western blotting using an antibody raised against a synthetic rat peptide, but it is preferentially found in the insoluble fraction in rat tissues including skeletal muscle (Ma et al., 2001). In skeletal muscle fibres from young mice, calpain-10 is detected predominantly at the sarcolemma and nucleus and an increase in intracellular calcium concentration leads to an increase in the nuclear localisation of calpain-10 in rat cultured lens epithelial cells (Ma et al., 2001).

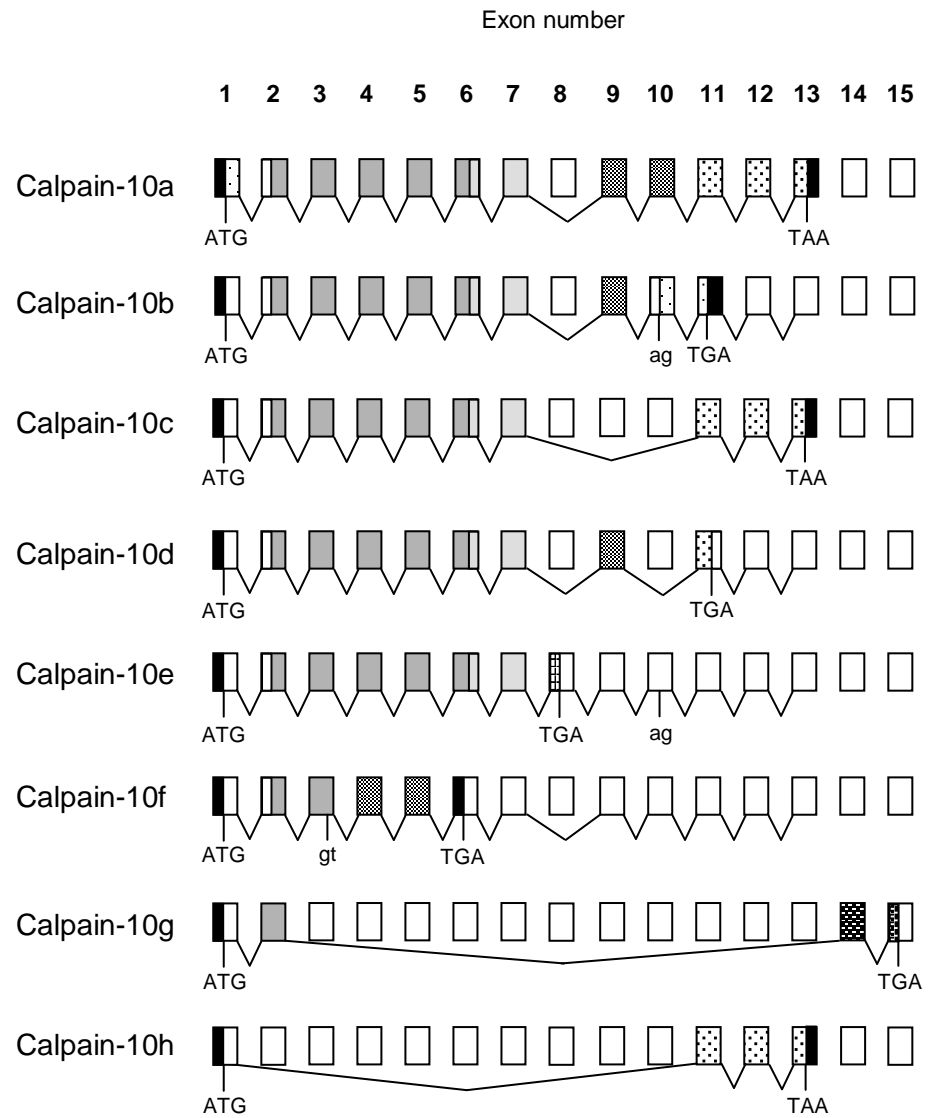


Figure 1.3 Exon structure and splicing pattern for human calpain-10 transcripts.

The calpain-10 gene expands through 15 exons and a complex pattern of alternative splicing generates up to eight isoforms, designated a – h. Each shaded box indicates the domain for which that exon, or part of the exon, encodes (see Fig 1.4). Adapted from Horikawa et al. (2000).

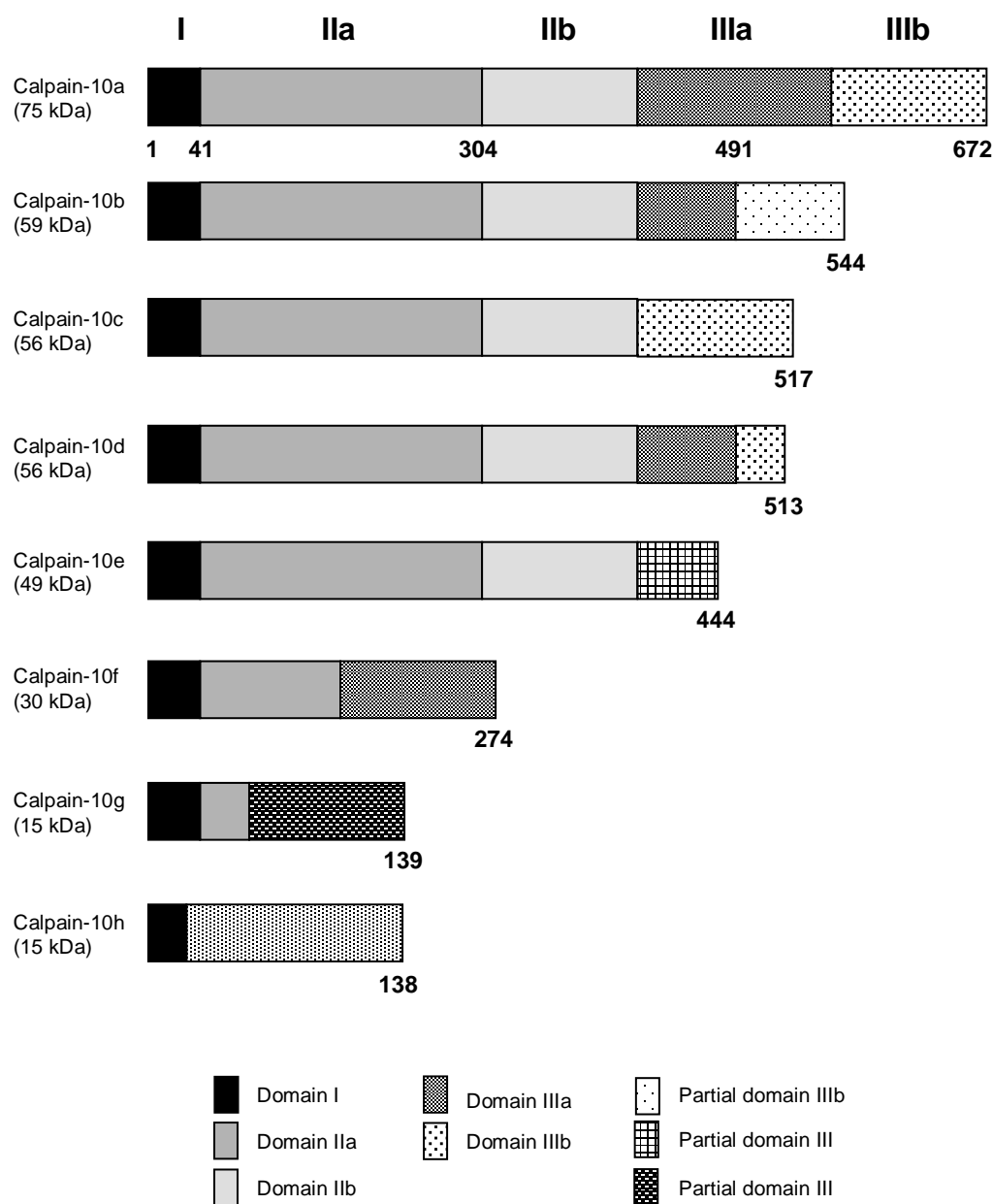


Figure 1.4 Domain structure of human calpain-10

Calpain-10 is made up of domain I, domain II and a repeat domain III. Alternative splicing (Fig 1.3) is thought to generate a number of proteins ranging in size from 672 (10a) to 138 (10f) amino acids. The numbers indicate the amino acid residue at the boundaries between the domains. Also shown is the predicted molecular weight of each calpain-10 isoform. Adapted from Horikawa et al. (2000).

1.9.2 Physiological functions of Calpain-10

Determining the function of calpain-10 has been and still is a tremendous challenge. Unfortunately, very little progress has been made in elucidating exactly what it is that calpain-10 does and this is particularly the case for some of the most important tissues for glucose homeostasis in humans, including the pancreas and skeletal muscle. The task has been complicated further by the apparent complex nature of the expression of calpain-10 isoforms and the potentially modifying influence of the identified risk SNPs. Perhaps the first question that arose in response to the initial findings that genetic variation in the calpain-10 gene influenced risk for type 2 diabetes concerned what influence these polymorphisms had, if any, on calpain-10 expression and subsequent function.

1.9.2.1 Single nucleotide polymorphisms and calpain-10 function

The SNP associated with an increased risk for type 2 diabetes (SNP-43) is located in intron-3 of calpain-10 (a region of DNA that is spliced out after transcription). Whilst this at risk allele has been linked to a wide variety of physiological phenotypes (see section 1.6), it is not clear how an SNP located in a non-coding region can influence the expression and/or function of a protein. The idea that polymorphisms located within introns can affect gene expression is not without precedent however. For example, a polymorphism in intron 1 of

COL1A1, encoding the binding site for the transcription factor Sp1, affects expression of this gene and is associated with reduced bone density and osteoporosis (Uitterlinden et al., 1998).

Experiments *in vitro* indicated that the intronic region surrounding SNP-43 occurred as a *cis*-acting element that was able to influence calpain-10 gene transcription (Horikawa et al., 2000). Using gel shift (EMSA) experiments, the authors were able to show that nuclear extracts could bind to the region around SNP-43 but that the G/A mutation had no effect on this binding. In follow up studies, Baier et al. (2000) inserted a 46 bp fragment of the SNP-43 region into a luciferase reporter construct and showed that the G allele had greater promoter activity than the A allele. Analysis of human skeletal muscle biopsy samples has added strength to the idea that SNP-43 affects gene transcription by showing that the at risk G/G genotype is associated with reduced calpain-10 mRNA expression when compared to the G/A and A/A group (Baier et al., 2000, Carlsson et al., 2005). It is interesting to note that the rare allele at SNP-44 is in perfect linkage disequilibrium with the missense mutation T504A and this mutation affects gene transcription (Evans et al., 2001).

1.9.2.2 Calpain-10 and glucose uptake

As mentioned above, the G/G genotype at SNP-43 was associated with reduced skeletal muscle calpain-10 mRNA expression in the skeletal muscle of Pima Indians (Baier et al., 2000). The level of skeletal muscle calpain-10 mRNA was also correlated with whole body glucose oxidative capacity suggesting the possibility that calpain-10 expression is associated with muscle glucose utilisation. More recent work has strengthened the finding that SNP-43 influences calpain-10 mRNA expression in skeletal muscle and also indicates that muscle calpain-10 mRNA expression is upregulated by insulin infusion following 24 h of lipid infusion in human subjects with impaired glucose tolerance (Carlsson et al., 2005).

Paul et al. (2003) showed that specific inhibition of calpain-10 using an inducible antisense oligonucleotide system decreased insulin stimulated glucose uptake in 3T3-L1 adipocytes by approximately 50%, whilst not affecting basal glucose uptake. Inhibition of calpain-10 expression also reduced insulin stimulated translocation of the specific glucose transporter GLUT4 to the plasma membrane of 3T3-L1 adipocytes, and reduced insulin stimulated actin reorganisation. Importantly, calpain was not shown to be required for the phosphorylation of critical signalling molecules involved in GLUT4 translocation, including insulin receptor β (IR β), insulin receptor substrate 2 (IRS2) and Akt/PKB (Paul et al., 2003).

The idea that calpains in general are involved in glucose transport in skeletal muscle has also been examined *in vitro*. In isolated muscle strips, non-specific calpain inhibition has also been shown to reduce insulin mediated glucose transport (Sreenan et al., 2001). Insulin stimulated 2-deoxyglucose uptake in the presence of the calpain inhibitors ALLM and E-64-d was considerably reduced (Sreenan et al., 2001). Attempting to control for the potential non-specific nature of these inhibitors, the authors showed that cathepsin inhibitor had no effect on insulin stimulated glucose transport. The reduction in glucose transport with calpain inhibition was matched by similar reductions in glycogen synthesis in isolated muscle strips (Sreenan et al., 2001). Again, this study, whilst informative does not provide direct evidence for a role of calpain-10 in insulin mediated glucose transport in skeletal muscle.

In mice overexpressing the specific calpain inhibitor calpastatin *in vivo*, skeletal muscle glucose uptake remained unchanged despite elevated levels of the glucose transporter GLUT4 protein, indicating insulin resistance relative to GLUT4 content (Otani et al., 2004). GLUT4 protein was also cleaved by m-calpain *in vitro* (Otani et al., 2004) suggesting that calpastatin overexpression inhibits calpain mediated GLUT4 turnover. Interestingly in the muscles of the calpastatin transgenic animals, the relative insulin resistance was associated with reduced expression of the critical insulin signalling protein Akt/PKB (Otani et al., 2004). Whilst these studies provide evidence that calpains

in general are involved in GLUT4 mediated glucose transport, it is not clear which specific calpain isoform is responsible for these effects. As mentioned previously calpastatin probably does not have any inhibitory action on calpain-10 but calpain-10 may participate in these pathways indirectly via interactions with other members of the calpain family.

1.9.2.3 Calpain-10 and skeletal muscle differentiation

Examination of the role of calpain-10 in muscle cell differentiation *in vitro* showed that as isolated human myoblasts differentiated to form multinucleated myotubes in culture, the expression of a 60 kDa immunoreactive band thought to represent a calpain-10 isoform or cleavage product increased (Logie et al., 2005). This finding is consistent with a role for calpain in the degradation of cytoskeletal/plasma membrane attachments during myogenesis (see 1.8.2.3). The potential mechanisms and protein substrates involved in calpain-10 mediated muscle differentiation are yet to be elucidated.

1.9.2.4 Calpain-10 and insulin release

Assessment of the role of calpain in insulin secretion from the pancreas has shown that long-term 48 h exposure to non-specific calpain inhibitors reversibly suppresses glucose-induced insulin secretion by 40-80% in mouse islets (Zhou et al., 2003). In contrast, short-term 4 h

exposure to non-specific calpain inhibitors increases the insulin secretory response to glucose as a result of accelerated exocytosis of insulin containing granules (Sreenan et al., 2001). Using an approach more specific to calpain-10 – stable overexpression of calpain-10 in a human pancreatic cell line (INS1), glucose responsive insulin secretion was enhanced in a calcium dependent manner (Marshall et al., 2005). Direct binding of a putative 54 kDa calpain-10 isoform to proteins important for the fusion of insulin containing granules with the plasma membrane was also reported (Marshall et al., 2005). One of these proteins (SNAP-25) undergoes calcium mediated proteolysis during the exocytosis of insulin containing granules and this was inhibited with the use of non-specific calpain inhibitors providing a possible link between calpain-10 and insulin secretion in INS1 cells (Marshall et al., 2005).

In pancreatic islets, it has also been shown that calpain-10 mediates ryanodine-induced apoptosis and may also be required for apoptosis induced by fatty acid palmitate and by hyperglycaemia (Johnson et al., 2004). Intracellular calcium stores play an important role in the regulation of apoptosis in many cell types. Ryanodine receptor calcium channels transmit calcium to the closely associated mitochondria and regulate mitochondrial ATP production and thus may be critical in calcium induced apoptosis in the β -cell. DNA laddering reflecting the organised cleavage of DNA and a marker of apoptosis were detected within 36 h of exposure of islets to a blocking concentration of ryanodine indicating that inhibition of calcium flux through ryanodine

receptors activates β -cell apoptosis (Johnson et al., 2004). Ryanodine was found to increase calpain activity and the calpain inhibitor ALLM blocked both ryanodine and palmitate induced β -cell apoptosis. Using the same transgenic mice as described above, the authors also showed that calpastatin overexpression reduced ryanodine and palmitate induced apoptosis. In mice, ryanodine increased calpain-10 mRNA in islets by 2.5 fold but did not affect μ - or m-calpain (Johnson et al., 2004). In islets from calpain-10 knock-out mice ryanodine failed to increase calpain activity and ryanodine and palmitate induced apoptosis was reduced (Johnson et al., 2004). Finally, hypoglycaemia, but not hyperglycaemia induced apoptosis was completely inhibited in calpain-10 knock-out mice, whereas it was increased in islets overexpressing calpain-10 (Johnson et al., 2004). These data suggest a role for calpain-10 in β -cell apoptosis pathways and indicate a possible link between glucose concentrations, apoptosis and calpain-10.

1.10 Calpain-3 as a type 2 diabetes gene

Recent evidence suggests that calpain-3 may influence the risk for type 2 diabetes. The NIDDM1 locus has been shown to interact with a gene on chromosome 15 (near CYP19) to further increase susceptibility to diabetes in Mexican Americans (Cox et al., 1999). The association of this region with type 2 diabetes has been confirmed in a Japanese population (Mori et al., 2002). This locus on chromosome 15 includes the calpain-3 gene, and given that calpain-3 is highly expressed in skeletal muscle which is a major site for insulin action, it is possible that an interaction between calpain-10 and calpain-3 in skeletal muscle may further increase the risk for type 2 diabetes.

1.11 Structure and function of calpain-3

1.11.1 Domain structure of calpain-3

The human calpain-3 gene is located on chromosome 15q15.1-q21.1 and covers a region of approximately 140 kb. The major product of the gene is encoded by 24 exons corresponding to a 3316 bp mRNA which is predominantly expressed in adult skeletal muscle (Sorimachi et al., 1989). Multiple alternative transcripts have been detected in human, mouse, rat and rabbit tissues, but usually with an expression level 100- to 1000-fold lower (Herasse et al., 1999; Kawabata et al., 2003). Some

of these transcripts are expressed from an additional alternative ubiquitous promoter known to be present in human and mouse genomes or from a lens-specific promoter detected in mouse, rat and rabbit genomes but absent from the human genome (Duguez et al., 2006).

Translation of the calpain-3 gene product leads to the production of a 94 kDa protein consisting of a short N-terminal region (domain I), a papain-like proteolytic domain (domains IIa and IIb), a C2-like domain (domain III) and a calcium-binding domain composed of five EF-hands (domain IV) as found in the ubiquitous calpains (Fig 1.5). Uniquely, calpain-3 possesses three sequences which are not found in any other calpains. These have been termed NS (N-terminal sequence), IS1 and IS2 (inserted sequences 1 and 2) (Fig 1.5) (Duguez et al., 2006). The NS sequence is a 20–30 amino acid N-terminal domain which is found in domain I and which corresponds to a regulatory propeptide found in other cysteine proteinases (Duguez., 2006). The IS1 sequence is a polypeptide of about 50 amino acids which is embedded in the proteolytic domain. This sequence contains three autolytic sites and, as a consequence, deleting this region renders calpain-3 inactive (Herassee et al., 1999). The IS2 sequence has also been demonstrated to be important for the control of the activity of calpain-3 (Herassee et al., 1999; Ono et al., 1998).

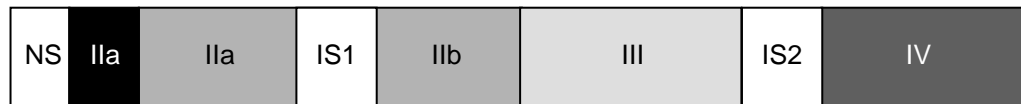


Figure 1.5 Calpain-3 protein domain structure.

The four domains including the specific calpain-3 insertion sequences (NS, IS1 and IS2) are shown. These insertion sequences are thought to control the stability of the calpain-3 protein.

1.11.2 Physiological functions of calpain-3

1.11.2.1 Calpain-3 autolysis and activation

Calpain 3 autolysis occurs rapidly in cells or poorly extracted muscle samples and possibly after physiological activation in living muscle (Sorimachi et al., 1993). It generates a small fragment of 30 kDa and a large C-terminal fragment, the size of which ranges from 60 to 55 kDa depending on the extent of autolysis (Kinbara et al., 1998). IS2 is a peptide of about 80 amino acids encoded by exons 15–16 and located between domain II and domain III. A basic sequence encoded by exon 15 seems to act as a nuclear translocation signal at least in human and

COS-7 cells (Sorimachi et al., 1993). IS2 has been demonstrated to be important in the control of the activity of calpain 3, as exon 16 deletion leads to loss of substrate proteolysis (Herasse et al., 1999).

Because of the rapid autolysis of calpain-3, it has so far been impossible to obtain crystals of the full molecule. However, a three dimensional model of calpain-3 based on the known structure of m-calpain shows that the proteolytic domain can be subdivided into two globular subdomains (domain IIa and IIb), forming a catalytic cleft at their interface (Jia et al., 2001). As in ubiquitous calpains, domain III of calpain-3 fits a C2 motif. In this model, IS1 and IS2 have been structured as loops protruding out of the globular core structure (Jia et al., 2001).

1.11.2.2 Calpain-3 and muscle growth

Calpain-3 in skeletal muscle is bound to titin/connectin at the N2A and M-line regions, as shown with the use of the yeast two hybrid system (Sorimachi et al., 1995). Experiments *in vitro* have shown that calpain-3 is capable of cleaving numerous myofibrillar proteins including titin itself, filamin C, vinexin, exrin and talin (Taveau et al., 2003; Guyon et al., 2003; Kramerova et al., 2004). It is currently not clear whether calpain-3 is able to cleave these proteins *in vivo* but the close proximity of these proteins to calpain-3 would indicate such an interaction. Evidence that calpain-3 is critical for muscle function, however, comes

from the finding that loss-of-function mutations within the calpain-3 gene cause limb girdle muscular dystrophy type 2A (LGMD2A) (Richard et al., 1995). It is thought that it is the loss of substrate processing ability of calpain-3 that causes LGMD2A (Ono et al., 1998), although the exact physiological substrates are not known. It is clear that calpain-3 is not essential for the initial building of functional skeletal muscle as patients with LGMD2A develop normally and the mean age of onset of the disease is in the second decade of life (Duguez et al., 2006). It has also been demonstrated convincingly that calpain-3 is not critical for myoblast proliferation and fusion as the expression of full length calpain-3 occurs after muscle innervation and fusion *in vivo* (Fougerousse et al., 1998). Denervation of healthy adult mouse muscle leads initially to the disuse of the muscle and, later, atrophy and this is associated with reduced calpain-3 mRNA (Stockholm et al., 2001). In humans, disuse atrophy is similarly associated with reductions in muscle mass and calpain-3 mRNA levels (Jones et al., 2004). Together these results indicate that the function of calpain-3, which is directed towards the proteolysis of specific substrates, seems to be important in fully differentiated fibres and its absence leads to degeneration and death of the fibre, most likely as a result of the deregulation of sarcomere remodelling (e.g. Kramerova et al., 2005).

1.11.2.3 Calpain-3 and signal transduction pathways in muscle

The idea that calpain-3 participates in signalling pathways in skeletal muscle came from the observation that in LGMD2A patients, myonuclear apoptosis in the deltoid muscle was correlated with altered subcellular distribution of Inhibitor κ B- α (I κ B α) and Nuclear Factor κ B (NF- κ B) (Baghdiguian et al., 1999). More specifically, calpain-3 deficiency was associated with accumulation and nuclear translocation of I κ B α and increased sarcoplasmic localisation of NF- κ B (Baghdiguian et al., 1999). Degradation of I κ B α unmask the nuclear localisation signal on the p65 subunit of NF- κ B allowing it to move to the nucleus and activate the expression of genes involved in cell survival. Purified recombinant I κ B α was completely degraded in a calcium dependent manner by the ubiquitous calpains *in vitro* (Baghdiguian et al., 1999). In a cell expression system, co-expression of calpain-3 and I κ B α led to a significant reduction in the levels of I κ B α (Baghdiguian et al., 1999). These results indicate that reduced cleavage of I κ B α , as a result of reduced calpain-3 activity, leads to increased NF- κ B sequestration in the cytoplasm and increased apoptosis in the skeletal muscle of LGMD2A patients. Interestingly, this pathway may also provide a possible link between calpain-3 and insulin resistance as NF- κ B has been linked to serine phosphorylation of IRS1 and a downregulation of insulin mediated glucose uptake (see 1.3.1.2).

1.11.2.4 Calpain-3, insulin resistance and type 2 diabetes

Calpain-3 plays an important role in the structure and function of skeletal muscle, as outlined above. Interestingly it was recently shown that the calpain-3 gene overlaps that of the gene encoding α -glucosidase C, a gene which is important for glycogen breakdown (Kawabata et al., 2003). These data suggest the possibility that both genes affect one another's transcription (Kawabata et al., 2003). Only one study has investigated calpain-3 expression in relation to type 2 diabetes related traits. Walder et al. (2003) reported that reduced calpain-3 mRNA expression was associated with phenotypes related to obesity and insulin resistance (e.g., increased abdominal fat mass and circulating blood glucose) in both humans and *Psammomys obesus*, a polygenic animal model of obesity and type 2 diabetes. Moreover, skeletal muscle calpain-3 expression has been shown to be affected in states of insulin resistance; for example, fasting in lambs (Illian et al., 2001) and chicks (Nakashima et al., 2005) leads to an increase in calpain-3 expression.

1.12 Summary and aims

Early studies on calpain-10 provided strong evidence to suggest that calpain-10 was associated with an increased risk for type 2 diabetes in a number of populations. Since these findings, some studies have attempted to identify possible mechanisms whereby calpain-10 may influence carbohydrate metabolism. These studies have provided only limited evidence *in vitro* to suggest that calpain-10 is directly involved in glucose utilisation. Moreover, in humans, there is currently no evidence to suggest that calpain-10 plays a direct role in glucose utilisation in human skeletal muscle *in vivo*.

The aim of this thesis was to examine the effect of altering skeletal muscle insulin sensitivity in healthy humans on the expression of calpain-10 and -3. A number of physiological interventions were performed in humans *in vivo* to manipulate insulin sensitivity in both a positive and a negative fashion and the expression of calpain-10 and -3 mRNA and protein was determined in skeletal muscle biopsies. In the first such study, healthy human subjects underwent short-term 48 h fasting and it was hypothesised that this intervention would induce transient insulin resistance, which could be restored by 24 h of high carbohydrate refeeding. It was envisaged that this fasting induced insulin resistance would be accompanied by a reduction in calpain-10 mRNA and protein expression in skeletal muscle and would therefore

provide evidence that calpain-10 was functionally linked to, rather than associated with, states of insulin resistance.

In the second and third studies, subjects underwent a one week high fat diet and an Intralipid infusion, respectively and it was hypothesised that an increase in fat availability in both studies would induce skeletal muscle insulin resistance. It has been shown previously that calpain-10 is regulated by lipid infusion in humans and therefore these studies had the joint aim of assessing the role of elevated FFA and insulin resistance on calpain-10 expression in skeletal muscle. It was also hypothesised that calpain-3 expression levels would be reduced in these interventions as calpain-3 expression has previously been negatively correlated with FFA and insulin resistance.

In the following chapter, a single bout of moderate intensity cycling exercise was employed as an intervention with the aim of increasing insulin sensitivity in an insulin independent manner. Previous studies have suggested a link between exercise, albeit accompanied with muscular damage, and calpain activation *in vivo*. It was hypothesised that calpain-10 expression would be induced in these states, possibly as a result of the increased calcium release that is seen during exercise. It was also hypothesised that calpain-3 expression would be activated during exercise and that this may be seen as a characteristic pattern of autolysis on Western blots of calpain-3 protein in skeletal muscle.

In the final chapter of this thesis, the expression of calpain-10 was investigated in the skeletal muscle of insulin resistant type 2 diabetic patients. Again, it was hypothesised that calpain-10 expression would be downregulated in the skeletal muscle of these patients. This finding would provide conclusive evidence of an association between calpain-10 and type 2 diabetes. Also in the final chapter, the role of variation in the calpain-10 gene on carbohydrate oxidation and glucose disposal was investigated by combining results from all of the previous studies. It was expected that the at-risk variation in the calpain-10 gene would be linked to reduced insulin stimulated glucose disposal and carbohydrate oxidation.

2 General Methods

2.1 Chemical reagents and equipment

All general laboratory chemicals were obtained from the following suppliers, unless otherwise stated: Fisher Scientific (Loughborough, Leicestershire, UK), Sigma Aldrich Chemicals (Poole, Dorset, UK), Promega (Southampton, UK) or GE healthcare (Little Chalfont, UK). Laboratory buffers were made up with distilled water or ultra-pure Milli-Q Elix water (Millipore Corporation, Watford, UK) where required. Water used for nucleic acid work was always DNase and RNase free, and was purchased from Sigma Aldrich. The majority of molecular biology reagents and enzymes were purchased from Promega and/or New England Biolabs (Hitchin, Hertfordshire, UK). Real-time PCR consumables were purchased mostly from Applied Biosystems (Warrington, UK) with additional materials purchased from Invitrogen (Paisley, UK) or ABgene (Epsom, Surrey, UK) where required.

Equipment which was used but is not specified in the text was as follows. Centrifuges used were the Biofuge 13 (Heraeus Instruments/Kendro, Hertfordshire, UK), the Hawk 15/05 (MSE, London, UK) and the Centraur swinging bucket centrifuge (MSE, London, UK). Instruments used for protein quantification were the Spectra Max 190 (Molecular Devices Ltd., Wokingham, UK), the Ultraspec Plus (Biochrom

Ltd, Cambridge, UK) and the 680 XR microplate reader (Bio-Rad, Hemel Hemstead, UK). For DNA quantification either the GeneQuant Pro (Biocom Ltd., Cambridge, UK) or the NanoDrop ND-1000 (Wilmington, USA) was used. The machines used for PCR were the Peltier Thermal Cycler-200 (MJ Research Inc, Massachusetts, USA) and the Mastercycler (Eppendorf, Cambridge, UK). For real-time PCR the same ABI Prism 7700 (Applied Biosystems, Warrington, UK) system was always used.

For running large (200 mm x 100 mm) sodium dodecyl sulphate polyacrylamide gel electrophoresis gels (SDS-PAGE), a dual plate unit was used (Fisher or GE healthcare). Western blotting of large protein gels was performed using a Trans-blot fitted with plate electrodes (Bio-Rad). For small mini protein gels (80 mm x 50 mm), the Mini-Protean II system (Bio-Rad) was used for gel running and Western blotting. Horizontal DNA agarose electrophoresis was carried out using the Sub-Cell system (Bio-Rad).

2.2 Human studies

Each individual chapter described in this thesis represent a distinct study or group of studies. Inevitably each study involved a distinct set of techniques, whilst a number of techniques were common to each study. The protocols common to all chapters are described in this chapter and those that are more specific to individual chapters are described in the relevant chapter.

2.2.1 Human Volunteers

All subjects were recruited from the students and staff of the University of Nottingham or from the local population. Posters were used to advertise the studies and these were usually placed in and around the university campus. Prospective studies were also advertised on the website of the Centre of Integrated Systems Biology and Medicine (<http://www.nottingham.ac.uk/cisbm/>). The experimental protocol was described to potential subjects in detail and time was allocated to allow for the individual to ask any questions that they may have had regarding any aspect of the study. Following this meeting, subjects were then asked to give written informed consent and complete a basic medical screening involving a general health questionnaire and measurements of height and weight using a stadiometer and an electronic balance, respectively. Blood pressure and ECG readings were taken and a blood sample was collected from an antecubital vein for analysis of full

blood count and markers of renal and hepatological function (QMC). All studies were approved by the University of Nottingham Medical School Ethical Committee.

2.2.2 Blood sampling and analysis

On study visits, arterialised-venous (a-v) blood samples were obtained from the dorsal surface of a superficial vein in the non-dominant hand. The hand was placed in a small chamber which was heated to approximately 55°C to arterialise the venous drainage of the hand. After heating the hand for approximately 15 min, local anaesthetic was applied to the skin (1% lignocaine hydrochloride, Antigen Pharmaceuticals Ltd., Roscrea, Ireland) and a 21 gauge cannula was inserted retrogradally into a vein. The hand was then returned to the chamber where it remained for the duration of the study. In cases where exercise was in progress (**chapter 6**), subjects were asked to place their hand into the heated chamber approximately 5 min before sampling began. An isotonic saline drip (0.9% sodium chloride BP, Baxter Healthcare Ltd., Thetford, UK) was used to keep the cannula clear and accessible at all times. Blood samples were withdrawn via a three-way tap with the first 2 ml being discarded to avoid dilution with saline.

2.2.2.1 Determination of blood glucose

Following blood sampling an aliquot of approximately 25 µl of whole blood was aspirated into an automated glucose analyser (YSI 2300 STAT plus, Yellow Springs, OH, USA) for determination of whole blood glucose concentration. The YSI STAT 2300 uses a steady state measurement methodology, where membrane based glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (H₂O₂) (Twomey et al., 2004; Astles et al., 1996). The glucose analyser was calibrated prior to each experiment and at regular intervals during the experiment, as described by the manufacturer.

2.2.2.2 Determination of serum insulin concentrations

For collection of serum, an aliquot of whole blood was collected into 5 ml tubes (BD Vacutainer, SSTII advance) and allowed to clot for at least 20 min, following which the serum was collected by centrifugation at 3,000 g for 15 min and stored at -80°C. Serum insulin was determined at a later date using a solid phase (coat-a-count) radioimmunoassay (Diagnostic Products Corporation, California, USA) according to the manufacturers instructions (**appendix 1**).

2.2.2.3 Determination of plasma free fatty acids (FFA)

For collection of plasma, an aliquot of whole blood was also collected into 6 ml tubes (BD vacuater, LH 102 I.U) containing 30 µl of 200 mM EGTA (to inhibit lipolysis) and lithium heparin, and plasma was collected by centrifugation at 3,000 g for 10 min and stored at -80°C. Plasma FFA concentrations were determined using the NEFA C Kit (WAKO Chemicals, Germany) according to the manufacturers instructions (**appendix 1**).

2.2.2.4 Determination of ketones

Whole blood β-hydroxybutyrate was measured in perchloric acid (10%) treated whole blood samples, according to the method of Williamson et al. (1962) (**appendix 1**). This assay was performed by Sally Cordon at the School of Biomedical Sciences, University of Nottingham.

2.2.2.5 Determination of glucagon concentrations

Plasma samples from whole blood were prepared as described above and plasma glucagons was analysed using a double-antibody glucagon test kit (Diagnostic Products Corporation, California, USA) according to the manufacturers instructions (**appendix 1**). This assay was

performed by Dr. K. Chokkalingam at the Queens Medical Centre, Nottingham.

2.2.2.6 Genomic DNA extraction

In all studies undertaken, a baseline blood sample was collected during the first experimental visit for the extraction of genomic DNA and for the determination of each individuals genotype at various calpain-10 SNPs (**chapter 1, 1.6**). The DNA was extracted from 300 µl of whole blood using the Wizard Genomic DNA purification kit (Promega). Three hundred microliters of blood was added to 900 µl of cell lysis solution and inverted 5-6 times to mix the contents well before being incubated at room temperature for 10 min to lyse the cells. The sample was then centrifuged at 13,000 g for 20 seconds at room temperature, the supernatant removed and the remaining white blood cells were resuspended in approximately 20 µl of residual liquid from the supernatant. Three hundred microliters of nuclei lysis solution was added to the cells and pipetted gently to lyse the white blood cells. Protein was precipitated with 300 µl of protein precipitation solution and collected by centrifugation at 13,000 g for 3 min. The supernatant was transferred to a clean eppendorf containing 300 µl of isopropanol and the tube was inverted repeatedly until white DNA strands became visible (usually 5-10 inverts). At this point the DNA was collected by centrifugation at 13,000 g for 1 min, washed with 75% ethanol and resuspended in 100 µl of H₂O. DNA was quantified and the

determination of each subjects genotype at a number of loci is described below (see 2.5).

2.2.3 Urine sampling and analysis

Urine samples were collected during each study visit in **chapter 4** and **chapter 5** and stored in 5 ml aliquots at -20°C in 10% thymol until analysis. For determination of protein oxidation in **chapter 4**, urine nitrogen was determined using the Kjeldahl method and was performed at the University of Surrey, UK. In **chapter 5**, protein oxidation was estimated from urea excretion as most urinary nitrogen (>80%) is in the form of urea. Urea concentrations were determined using a commercially available kit (Randox Laboratories, UK) according to the manufacturer's instructions (**appendix 1**) and this assay was performed by Dr. K. Chokkalingam, Queens Medical Centre, Nottingham. It was assumed that for each gram of nitrogen excreted in the urine, 6.04 litres of O_2 were consumed and 4.89 litres of CO_2 were produced and results were normalised for protein content according to equations (1) and (2) below.

2.2.4 Indirect calorimetry

Resting metabolic rate (RMR) and substrate oxidation rates were determined *in vivo* from gaseous exchange data obtained using a

ventilated hood system. RMR was measured by using an open-circuit indirect calorimeter (Gas Exchange Measurement (GEM) system, Nutren Technology Ltd, Manchester, United Kingdom). After a warm-up period of 30 min, a reference gas (5% CO₂ and 95% O₂) was used to calibrate the oxygen and carbon dioxide analysers. Ingoing and outgoing air were analysed for oxygen and carbon dioxide composition every minute during each period of measurement, which was 20 minutes. Readings from the metabolic monitor were collected every minute with a personal computer, and this allowed for minute by minute calculations of the respiratory exchange ratio (RER) and of metabolic rate using the Weir equation (Weir, 1949). Substrate oxidation rates were estimated from $\dot{V}O_2$ and $\dot{V}CO_2$ values using stoichiometric equations as described in detail by Frayn (1983). The calculations used for the determination of carbohydrate (COX) and fat oxidation (FOX) based on O₂ consumed and CO₂ produced are summarised in equations 1 and 2, where n grams of urinary nitrogen are being excreted per minute.

$$COX = 4.55 \dot{V}CO_2 - 3.21 \dot{V}O_2 - 2.87 n \quad (1)$$

$$FOX = 1.67 \dot{V}O_2 - 1.67 \dot{V}CO_2 - 1.92 n \quad (2)$$

2.2.5 Peak oxygen uptake test ($\dot{V}O_2$ max)

In **chapter 6** each subject was asked to complete an incremental exercise test on a bicycle ergometer prior to the study to establish their maximal oxygen uptake, or $\dot{V}O_2$ peak. The subjects warmed up for a few minutes at low intensity before the workload was increased to 100 W and then subsequently by 25 – 50 W every 3 min depending on their estimated fitness status. During the cycling, subjects breathed through a mouthpiece and valve and $\dot{V}O_2$ and the rate of carbon dioxide production ($\dot{V}CO_2$) were measured by indirect calorimetry (Vmax, Sensor Medics, Yorba Linda, CA). Subjects were asked to maintain a constant speed throughout the test (~ 80 rpm) and were deemed to have reached their $\dot{V}O_2$ peak when they were no longer able to maintain this speed as the workload increased. Heart rate (Polar A1, Polar Electro Oy, Kempele, Finland) and perceived exertion (Borg and Linderholm, 1970) were monitored throughout to confirm that the subjects had reached their peak value. Linear regression was used to estimate the work load required for a given percentage of the $\dot{V}O_2$ peak. During studies in which bicycling exercise was a component (**chapter 6** only), measurements of $\dot{V}O_2$ and $\dot{V}CO_2$ were taken using the same equipment at regular intervals during the exercise period for determination of oxygen uptake and for calculations of substrate oxidation rates as described above.

2.2.6 The hyperinsulinaemic-euglycaemic clamp technique

In all experiments (except **chapter 3**), to quantify insulin sensitivity subjects underwent a hyperinsulinaemic-euglycaemic clamp essentially as described by DeFronzo et al. (1979). The clamps varied in duration, ranging from 4 h (**chapter 4** and **chapter 6**) to 6 h (**chapter 5**), but in all cases involved the simultaneous intravenous infusion of a predetermined fixed dosage of insulin and a variable rate glucose infusion. The insulin infusion rate was aimed at achieving a physiologically high serum insulin concentrations of approximately 80 mU/L, whilst the blood glucose concentration was 'clamped' at approximately 4.5 mmol/L to represent normal fasting values.

After the subjects reported to the laboratory, their weight was recorded and they were allowed a short rest period before a cannula was inserted into the non-dominant hand for the sampling of arterialised blood (see 2.2.2), and an additional cannula was inserted for the infusion of insulin and glucose. The insulin infusion rate (ml/hr) was calculated by multiplying the F value $[(\text{body surface area}/\text{insulin syringe conc.}) \times (\text{desired insulin clamp conc. (mU/m}^2)/40)]$ by the rate constant (DeFronzo et al., 1979). At 0-2 min, a bolus of insulin was given, which was gradually reduced every 2 min until 10 min into the clamp, where it was kept constant throughout the remainder of the clamp. The glucose infusion (2 mg/kg/min) was started at 4 min, and increased to 2.5 mg/kg/min at 10 min. During the remainder of the clamp procedure,

arterialised blood samples were taken every 5 min for the immediate determination of blood glucose concentrations. If the blood glucose concentration deviated from 4.5 mmol/l, then the glucose infusion rate was adjusted accordingly. At the end of the clamp period, the infusion of insulin was stopped and the subject was immediately fed a large carbohydrate rich meal. During this period, the infusion of glucose was maintained but was reduced slowly to allow the subjects blood concentrations to return to normal postabsorptive values, at which point the infusion of glucose was stopped, all catheters were removed, and the subject was allowed to leave the laboratory.

2.2.7 Muscle Sampling and analysis

Muscle samples were obtained from the vastus lateralis muscle by an experienced clinician using the needle muscle biopsy technique (Bergström, 1962). Briefly, subjects rested in a supine position and the skin overlaying the area to be sampled was shaved, if required, and thoroughly cleaned with iodine solution and then anaesthetised (1% lignocaine hydrochloride, Braun Ltd., Melsungen, Germany). After allowing a short time for the anaesthetic to take affect, a small scalpel blade was used to make a 0.5 cm incision through the skin. A biopsy needle was then inserted through the incision and a small sample of muscle (~ 50-100 mg) was removed. Muscle biopsy samples were snap frozen and stored in liquid nitrogen until further use.

2.2.7.1 Skeletal muscle total RNA extraction

Total RNA was extracted from ~10-30 mg of skeletal muscle biopsy tissue using Trizol reagent (Invitrogen, Paisley, UK) which is an adaptation of the guanidine isothiocyanate method developed by Chomczynski and Sacchi. (1987). Each piece of frozen muscle was homogenised on ice with a power homogeniser (PowerGen 700, Fisherbrand, Fisher Scientific) in 800 µl of Trizol reagent and 20 µl of glycogen (10 µg/µl) for 2 x 15 seconds with the sample being placed on ice for 30 seconds in between. The sample was allowed to stand at RT for 5 minutes before the addition of 160 µl of chloroform:isoamyl alcohol (49:1). The sample was then mixed by inversion for 20 seconds and then vortexed briefly and allowed to stand at RT for 2 min before centrifugation at 12,000 g for 15 min at 4°C. The aqueous phase was then transferred to a fresh tube and 400 µl of ice cold isopropanol was added and the samples were placed at -20°C overnight to precipitate the RNA. The next day, the RNA was pelleted by centrifugation at 12,000 g for 15 min at 4°C. The supernatant was removed and the pellet was washed with 800 µl of ice cold 75% ethanol and the samples were centrifuged again at 10,000 g for 10 min at 4°C. The supernatant was decanted and samples were placed over tissue paper for approximately 5 min to drain off any remaining ethanol. Following a brief pulse in the centrifuge, any last remaining ethanol was removed using a pipette, and the RNA pellet was dissolved in 30 µl of RNase

free H₂O on ice and the RNA solution was stored at -80°C. Integrity of extracted RNA was always checked on agarose gels (see 2.3.4).

2.2.7.2 RNA quantification

The concentration of extracted total RNA was quantified using the Ribogreen RNA quantification kit (Molecular Probes, Invitrogen, Paisley, UK). Two microliters of the RNA was diluted 10-fold in RNase free H₂O and 10 µl of this was added to 490 µl of 1 x TE buffer and 500 µl of ribogreen solution diluted 1:200 in 1 x TE. The fluorescence (excite at 480 nm, emit at 520 nm) of each sample was measured with a F-2000 fluorescence spec (Hitachi instruments Inc., Naperville, USA). For the standard curve, the supplied ribosomal RNA stock solution was diluted 1:50 in 1 x TE and increasing volumes of the diluted standard was added to 1 x TE to a final volume of 500 µl to give a standard curve ranging from 0-50 ng. Five-hundred microliters of the ribogreen solution was then added to each standard and measured as described above.

2.2.7.3 First strand cDNA synthesis

Reverse transcription was carried out on 0.5 µg total RNA essentially as described in Parr et al. (2001). Each RNA sample was diluted to 0.05 µg/µl and 10 µl of this was added to 1 µl random hexamer primers (Promega) and 4 µl H₂O and incubated at 70°C for 5 min in a

Mastercycler thermocycler (Eppendorf, Hamburg, Germany). The samples were immediately placed in ice before the addition of 10 mM dNTPs, 0.5 μ l RNase inhibitor, 5 μ l MMLV reverse transcriptase buffer and 1 μ l of MMLV reverse transcriptase (all Promega) in a final volume of 25 μ l. In the same thermocycler, the samples were incubated at RT for 10 min followed by 42°C for 1 h and 70°C for 15 min.

2.2.7.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Following reverse transcription, the first strand cDNA was always diluted fourfold (i.e. 75 μ l of RNase free H₂O was added to 25 μ l first strand cDNA solution). Quantification of reverse transcribed cDNA was performed in real time using an ABI 7700 Sequence Detection System as described above. Human cDNA sequences were obtained from GenBank and primers and dual-labelled fluorescent oligonucleotide probes (5'FAM and 3'TAMRA) were designed (appendix 2) using Primer Express version 2.0 software (Perkin-Elmer, Norwalk, CT). Real-time PCR was performed using PCR Universal Master Mix (Applied Biosystems, Warrington, UK). Each reaction contained 5 μ l cDNA template, 12.5 μ l PCR Universal Master Mix, 300 nM primers and 125 nM dual labelled fluorescence probe in a reaction volume of 25 μ l. Each RT-PCR reaction was performed in triplicate and all results were normalised to α -actin mRNA expression using the standard curve method. In **chapter 7**, results were normalised to HMBS mRNA

expression using the standard curve method. The primers and probes used in this thesis for qRT-PCR are listed in **appendix 2**.

2.2.7.5 Skeletal muscle protein extraction

Approximately 10-30 mg of tissue was homogenised for 30 s in 10 volumes of extraction buffer A containing 20 mM Tris-HCL, 5 mM EDTA, 2 mM DTT (pH 7.5) containing a cocktail of protease inhibitors (P8340, Sigma, Dorset, UK) (**chapters 3 and 4**). In later chapters (**chapters 5 and 7**), muscle tissue was homogenised in 10 volumes of extraction buffer B containing (50 mM HEPES, 10 % glycerol, 1 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 150 mM NaCl and 1% Triton X-100, pH 7.5) to improve the solubility of calpain-10 in particular (2.2.7.6). Whole muscle lysates were centrifuged at 13,000 g for 20 min at 4°C to pellet insoluble material and equal volumes of 2 x Laemmli sample buffer (20% glycerol, 125 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 100 mM DTT (or 100 mM β-mercaptoethanol), 0.02% (w/v) bromophenol blue) was added to the supernatant fraction. Protein pellets that remained from extractions with buffer A and B were resuspended in approximately 400 µl of 2 x Laemmli sample buffer supplemented with 8 M urea and 2 M thiourea and incubated overnight at room temperature with gentle shaking in order to fully dissolve the protein pellet.

2.2.7.6 Comparison of protein extraction buffers

To compare the extractability of calpain-10 with buffer A and buffer B, a small pilot experiment was performed. Briefly, baseline muscle biopsy protein was extracted from 5 random subjects with buffer A and the buffer B using identical procedures as described above. Samples for analysis were taken at each stage of the extraction process: i.e. after homogenisation (designated whole homogenate), and after centrifugation (supernatant and insoluble pellet). Protein samples were quantified and equal quantities of protein were run on SDS-PAGE gels, transferred to nitrocellulose membranes and immunoprobed for calpain-10 exactly as described below. As shown in Fig 2.1, replacing the extraction buffer to one that included a non-ionic detergent resulted in significant improvements in the solubility of calpain-10 protein. The quantity of calpain-10 protein in the supernatant fraction using buffer A was very low and was almost undetectable, whereas with buffer B quantification of calpain-10 in the supernatant fraction was clearly more viable.

When using buffer A, it is clear from the Western blot images that the majority of the total calpain-10 pool is being measured as very little immunoreactive calpain-10 is detected in the supernatant fraction following centrifugation, leaving the remainder in the insoluble fraction. In the case of buffer B, Fig 1 shows that most of the calpain-10 detected in the whole homogenate fraction is also detected in the supernatant

following centrifugation, again indicating that the majority of calpain-10 protein is indeed being detected. As an index of the biological and analytical variation, the coefficient of variation (CV) for the Western blot determination of calpain-10 was calculated for the relevant fractions in the above experiment. In the pellet remaining from buffer A and in the supernatant from buffer B, the CV for calpain-10 determination was 6.9% and 12.1%, respectively.

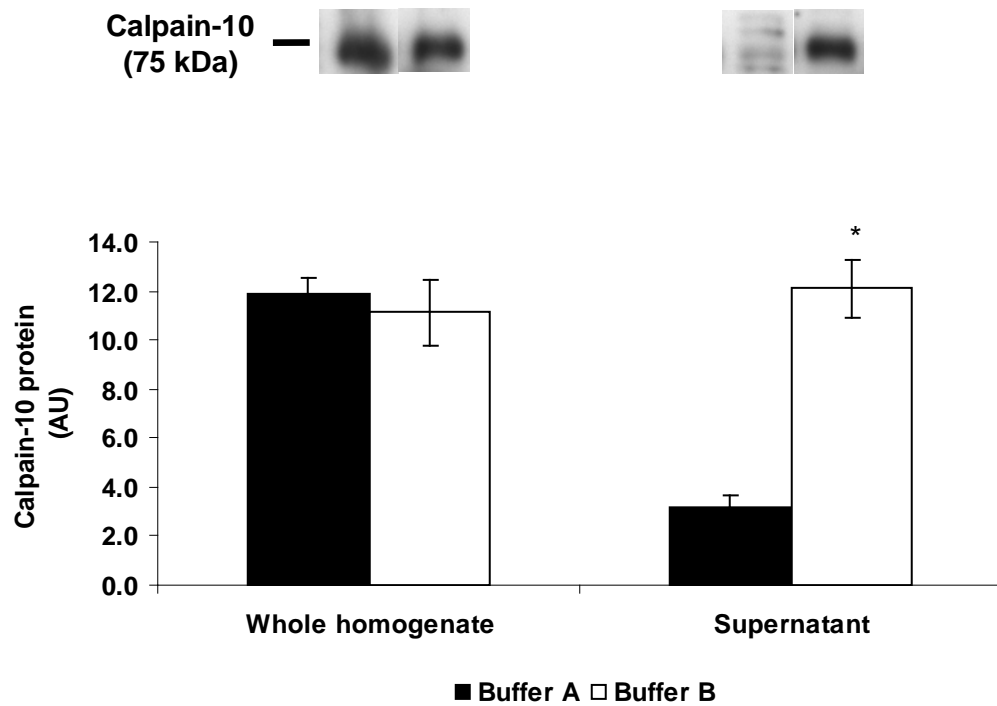


Figure 2.1 The effect of different extraction buffers on calpain-10 protein solubility in skeletal muscle (n = 5).

Shown is a representative Western blot for calpain-10 (75 kDa) following extraction with either buffer A or buffer B. *P<0.001 vs. buffer A.

2.2.7.7 Protein quantification

The Lowry (Lowry et al., 1951) or BCA (Smith et al., 1985) method was used to quantify protein concentration following extraction with extraction buffer A or B, respectively. The Lowry method involves two reactions; the first reaction is a biuret reaction which reduces Cu^{+2} to Cu^{+1} and the second uses this Cu^{+1} to reduce the Folin-Ciocalteau reagent. This reaction is detectable in the range of 500 – 750 nm. A number of solutions were made up for the assay and mixed to form a reagent A and a reagent B. Reagent A contained 2% Na_2CO_3 in 0.1 M NaOH, 2% KNa tartrate and the copper reagent 1% CuSO_4 in a 10:1:1 ratio. Reagent B contained 0.1 M NaOH and Folin-Ciocalteau reagent in a ratio of 10:1. Samples for analysis were diluted ten fold in water and 5 μl of this was added to a well in a 96 well plate in duplicate. For the standard curve, BSA standard solutions were made ranging from 0 $\mu\text{g}/5 \mu\text{l}$ to 1 $\mu\text{g}/5 \mu\text{l}$ and 5 μl of each standard was added to wells in duplicate giving a standard curve range of 0 – 1 μg . Following this, 195 μl of 0.1 M NaOH was added to all sample and standard wells to give a final volume of 200 μl in each well. Fifty microliters of reagent A was added to each well and the plate was incubated at room temperature for 5 min. Fifty microliters of reagent B was then added to each well and the plate was incubated at room temperature for a further 15 min before it was read at 620 nm in a spectrophotometer plate reader.

When buffer B was used, the BCA (bicinchoninic acid) method of protein quantification (BCA protein assay kit, Pierce, Cramlington, UK) was employed to allow for any interfering substances that might have been present in the new buffer (e.g. Triton X-100). Standards were first prepared using BSA to give a range of 2000 – 25 µg/ml and 10 µl of each standard and sample were pipetted into a microplate well in duplicate. A working reagent was prepared from reagent A and reagent B (supplied in the kit) in a ratio of 50:1, and 200 µl of this was added to each well. The plate mixed briefly for 30 s and incubated at 37°C for 30 min before being read at 562 nm.

Protein pellets remaining from the extraction procedures were quantified using the 2D-Quant Kit (GE healthcare, Little Chalfont, UK) according to the manufacturers instructions. Again, a BSA standard curve of 0 – 50 µg/ml was prepared from the stock solution of 2 mg/ml by the addition of 0 – 25 µl to 1.5 ml eppendorf tubes in duplicate. Three microliters of each sample in 2 x laemmli buffer was added to 1.5 ml eppendorf tubes before 500 µl of precipitant was added to all sample and standard tubes; tubes were then briefly vortexed and incubated at room temperature for 2-3 min. Five-hundred microliters of co-precipitant was then added to each tube and tubes were again mixed briefly before being centrifuged at $\geq 10\,000\text{ g}$ for 10 min. Supernatants were carefully removed and proteins were dissolved in 100 µl of copper solution and 400 µl of water. To each tube was added 1 ml of working

colour reagent before samples were incubated at room temperature for 15-20 min and finally read at 480 nm.

2.2.7.8 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out essentially as described by Laemmli (1970). Protein samples were diluted in an equal volume of 2 x Laemmli sample buffer and each sample was then mixed and boiled for 5 min to ensure protein denaturation before being centrifuged briefly at maximum speed to collect any cell debris. Equal quantities of each sample, based on results from the quantification, were then loaded onto polyacrylamide gels consisting of a 5% (v/v) stacking gel and a main gel which ranged in concentration from 8-12% (v/v) depending on the application. Gels were run in SDS running buffer (25 mM Tris, 192 mM glycine and 1% (w/v) SDS at a constant voltage of 200 V for approximately 40 min. Prestained protein markers (Precision Plus Protein Standards, Bio-Rad, UK) were loaded onto each gel to accurately and consistently determine the size of each migrated band.

2.2.7.9 Western blotting

Western blotting was performed according to Towbin et al. (1979) and Burnette, (1981). The Western blot buffer used in this thesis contained 400 mM glycine, 25 mM Tris and 10% methanol. Whilst SDS-PAGE gels were running, nitrocellulose or polyvinylidene difluoride (PVDF) membranes (GE healthcare, Little Chalfont, UK) were cut to size, as were four pieces of 3MM paper. Blotting membranes, 3MM paper and western blot sponges were soaked in the western blot buffer for at least 20 min whilst the gel was running. In the case of PVDF membranes, these were hydrated for 15 sec in methanol and washed for 5 min in water before being soaked in western blot buffer. When the gel had finished running, it was soaked very briefly in western blot buffer (<5 min). Following this, the western blot sandwich was assembled; a sponge was laid onto one side of a blotting cassette, and placed on top of this was two sheets of 3MM paper, then the gel, followed by the membrane, the final two pieces of 3MM paper, and finally the remaining sponge. Care was taken not to introduce air bubbles into the sandwich. The cassette was clamped together and placed into a western blotting tank, taking care to ensure that the gel side of the assembly was orientated adjacent to the cathode. Western blotting was carried out for approximately 2 h at 150 – 350 mA depending on the size of the transfer tank used. Buffer was always kept cold during Western blotting with the use of cold packs or a super cooling coil contacted to the mains water supply.

2.2.7.10 Immunoprobings with calpain antisera

For the determination of calpain-10 protein expression, equal quantities (30 µg) of insoluble skeletal muscle protein extracts (**chapter 3 and 4**) or supernatant protein (**chapter 5 and 7**) were loaded onto 10% polyacrylamide gels and transferred to PVDF membranes as described above (see 2.2.7.9). The PVDF membrane was removed from the Western blot apparatus following transfer and briefly washed in methanol before being placed in Ponceau S stain (0.5% (w/v) Ponceau S in 5% (w/v) trichloroacetic acid) for approximately 1 min and membranes were visualised by destaining in methanol. This was to ensure 1) equal transfer efficiency across the gel and 2) that equal quantities of protein were loaded into each well. Ponceau S stain was removed by washing briefly in TBS-T (50 mM NaCl, 100 mM Tris, 1% (v/v) Tween 20). Non-specific binding was then blocked in 5% (w/v) non-fat dried milk (Marvel) in TBS-T for 1 h at room temperature before incubating overnight at 4°C with the primary antibody, diluted 1:1000 in 5% (w/v) Marvel TBS-T. The next morning, any unbound antibody was removed by washing with 1% (w/v) Marvel TBS-T for 12 x 5 min with fresh changes of solution each time. The membrane was then incubated for 1 h with the secondary antibody, which was anti-rabbit IgG linked to horseradish peroxidase (HRP) (GE healthcare, Little Chalfont, UK).

The membrane was developed with ECL Plus Western Blotting Detection Reagents (GE healthcare, Little Chalfont, UK). For this, a 40:1 ratio of reagent A to reagent B was mixed together, pipetted on to the membrane ($0.1\text{ml}/\text{cm}^2$) and incubated for 5 min at room temperature. The membrane was blotted using 3MM Whatman chromatography paper to remove the detection reagents and wrapped in cling film before being taped into an autoradiograph cassette. In the dark room, the membrane was exposed to autoradiography film (Hyperfilm ECL, GE healthcare) before being developed in 1 x Kodak X-ray developer solution (Calumet Photographic Company, Nottingham, UK) briefly washed in water and fixed in 1 x Ilford Hypam rapid fixing solution (Calumet Photographic Company). Western blot signals were quantified by scanning densitometry and analysed with Quantity-One Multi-Analyst software (Bio-Rad, Hertfordshire, UK).

For calpain-3, equal quantities (30 μg) of insoluble skeletal muscle protein extracts were run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes as described above (see 2.2.7.9). These membranes were stained with Ponceau S in the same way, except that water replaced methanol. The same detection procedure was used as described above for calpain-10 except that membranes were blocked overnight at 4°C before being incubated with a polyclonal antibody raised against a recombinant protein corresponding to domain-III of porcine calpain-3 (2.4), diluted 1:500 for 1 h. For the quantification of

calpain-3 autolysis products, the densities of the 60 and 58 kDa bands were added to the density of the 55 kDa band.

All results for calpain were normalised to the levels of desmin protein, which was quantified on each blot using a polyclonal anti-desmin antibody, diluted 1:500 (D-8281, Sigma, Dorset, UK). Briefly, membranes were stripped of detection reagents and primary and secondary antibodies using Restore Western Blot Stripping Buffer (Pierce, Rockford, USA) according to the manufacturers instructions and incubated overnight with the desmin antibody. The same detection procedure was used as described above for calpain-10.

2.3 Calpain-10 antisera

The generation of calpain-10 antisera was attempted in this thesis via the expression and purification of a small peptide corresponding to domain III of human calpain-10a. This was absolutely necessary as at the commencement of this project no calpain-10 antibodies were commercially available.

2.3.1 Human calpain-10 cDNA synthesis

An Expressed Sequence Tag (EST) containing a partial human calpain-10 cDNA sequence was used as a template for the generation of a 528 bp fragment of the human calpain-10 gene. A search of all available ESTs was carried out using the Genbank (<http://www.ncbi.nih.gov/Genbank/>) Express Sequence Tags database (dbEST). EST matches were aligned and the best match for the desired sequence was purchased from the I.M.A.G.E consortium (Integrated Molecular Analysis of Genomes and their Expression; <http://www.image.llnl.gov>) using MRC geneservice (<http://www.hgmp.mrc.ac.uk/geneservice/index.shtml>).

2.3.2 Amplification of human calpain-10 cDNA

Forward and reverse primers were designed to amplify a 528 bp region from within the EST based on sequences selected from domain III of human calpain-10a (see **chapter 1, Fig 1.4**). The forward primer was 5'-GGGGAGTTCTGGGTGGAGGAG-3' and corresponded to amino acids 299 – 305 of human calpain-10a and the reverse primer was 5'-GTCCCCAGCACCTTCCTG-3' and this corresponded to the non coding strand complimentary to amino acids 469 – 474 of human calpain-10a. Additional nucleotides were incorporated during synthesis at the 5' ends of the forward and reverse primer to facilitate cloning into the EcoRI (GGATCC) and BamHI (GAATCC) site of the pGEX-4T-1 fusion vector (GE healthcare, Little Chalfont, UK), respectively.

PCR reactions were performed in 50 µl final volumes containing the following reagents; 1 x Taq polymerase buffer (10 x stock: 200 mM Tris-HCl pH 8.4, 500 mM KCl, 50 mM MgCl₂), 10 µM each dNTP, 10 pmol forward and reverse primer, 2.5 U Ampli Taq Gold (Applied Biosystems) and approximately 30 ng plasmid DNA. PCR cycling conditions were 95°C for 5 min followed by 40 cycles of 95°C for 30 sec, 65°C for 1 min and 72°C for 1 min, and a final extension step of 72°C for 10 min.

2.3.3 DNA precipitation

DNA was concentrated and purified using an ethanol (EtOH)/sodium acetate (NaAc) method. Briefly, 2 volumes of 100% ethanol and 1/10 volume of 3M NaAc, pH 5.5, was added to the DNA sample and was left at -20°C for at least 30 min. The DNA was collected by centrifugation at 15,000 g for 20 minutes at 4°C. The pellet was washed in cold 75% EtOH and collected by centrifugation at 15,000 g for 10 min. The supernatant was decanted and the DNA was allowed to air dry briefly before being re-suspended in H₂O.

2.3.4 Horizontal agarose gel electrophoresis

DNA products were verified on 1% (w/v) agarose gels. Agarose was dissolved in a 1 x concentrated TAE buffer (2 M Tris, 50 mM EDTA, 5.7 % acetic acid), and boiled briefly to dissolve. After the mixture had cooled, it was poured into a casting module and a suitable comb was placed at one end for sample loading. Gels were run at 100 V for approximately one hour and removed from the casting module and stained in 0.5 µg/ml ethidium bromide (EtBr) for half an hour and briefly de-stained in H₂O. DNA bands were visualised using Bio-Rad's Gel Doc 2000 and Multianalyst program (Bio-Rad).

2.3.5 Restriction endonuclease digestion

Typically for analytical use, restriction digests were performed in 20 μ l volumes containing 2 μ l of substrate DNA (≥ 100 ng), 15.5 μ l H₂O, 2 μ l 10 x buffer and 0.5 μ l of enzyme (~ 5 units). For cloning experiments, larger amounts of DNA (vector and insert) were used and this meant scaling up the reactions volume considerably.

The purified calpain-10 cDNA was inserted into the pGEX-4T-1 vector using the BamHI/EcoRI restriction sites. First, 400 ng of the human calpain-10 cDNA was digested with BamHI and EcoRI in the same reaction, using 5 U of each enzyme in a 1 x multicores buffer (Promega) for 2 h at 37°C. After 1 h, an additional aliquot (5 U) of each enzyme was added. The pGEX-4T-1 vector was digested in two separate reactions; the vector was first linearised with EcoRI and then digested a second time with BamHI. In between digests, DNA was purified using the ethanol/sodium acetate method as described above (see 2.3.3) to remove non-compatible buffers. Reactions were performed in a final volume of 150 μ l containing 500 ng DNA and 50 U of either enzyme in a 1 x buffer (buffer E and H for BamHI and EcoRI, respectively). Again, the contents were mixed and placed at 37°C for 2 h, and an additional aliquot (50 U) of enzyme was added after 1 h. Following restriction digests, agarose gel electrophoresis (see 2.3.4) was used to estimate the quantity of both calpain-10 and pGEX-4T-1 DNA using molecular weight standards with known concentrations.

2.3.6 Ligation

In order to obtain the best efficiency for ligation, two vector:insert ratios of 1:1 and 1:3 were generally used. The following equation was used to calculate the quantity of insert DNA that was required for a given concentration of the vector DNA.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of} \frac{\text{insert}}{\text{vector}}$$

To ligate calpain-10 into the pGEX-4T-1 vector using a 1:1 ratio, approximately 4.2 ng of the insert DNA was added to a reaction mix containing 2 x ligation buffer (60 mM Tris-HCl, 20 mM MgCl₂, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol), 40 ng digested pGEX-4T-1 vector and 3 Weiss units of T4 DNA ligase. For a 1:3 vector:insert ratio, the amount of insert required was approximately 12.7 ng and the reaction mixture was adjusted accordingly. All ligation reactions were left overnight at 4°C.

2.3.7 Transformation

A 5 ml overnight culture of *Escherichia Coli* (*E.coli*) was back diluted into 20 ml of Lauria-Bertani-Miller (LB) media (10 g tryptone, 5 g yeast

extract and 10 g NaCl₂/l), and the culture was grown to the log phase (OD₆₀₀ = 0.4-0.5). Cells were then collected by centrifugation (3,000 g, 10 min) and resuspended in ice cold 0.1 M CaCl₂ using chilled, blunt ended pipette tips and left on ice for 1 h until competent. Approximately 10 ng of ligation mix was then added to 200 µl of cells and left on ice for a further 30 min. The cells were then heated to 42°C for exactly 2 min and placed back on ice for 20 min. Approximately 800 µl of LB media was then added to the cells and cells were allowed to grow at 37°C with shaking (200 rpm) for a minimum of 45 min. Cells were collected and 50-100 µl of resuspended cells were streaked onto LB-agar (15 g/l of LB media) plates containing ampicillin (50 µg/ml).

2.3.8 Plasmid purification and confirmation of positive transformants

Plasmid DNA was isolated using the GenElute plasmid miniprep kit (Sigma). Five millilitres of LB media (+ 100 µg/ml ampicillin) was inoculated with a single colony from an LB-agar plate and left shaking overnight at 37°C. The next day, cells were collected by centrifugation at 13,000 g for 1 min, the supernatant removed and cells resuspended in 200 µl of resuspension solution and lysed with the addition of 200 µl of lysis solution. The lysis solution was thoroughly mixed with the resuspended cells and left at room temperature for 5 min to ensure adequate lysis. Cell debris was precipitated with the addition of 350 µl of neutralisation/binding solution and collected by centrifugation at 13,000 g for 10 min. A GenElute miniprep column was then inserted

into a 2 ml eppendorf tube and the lysate was transferred to the column before it was centrifuged at 13,000 g for 1 min. The column flow-through was discarded and 750 µl of wash solution was added to the column and the column was again centrifuged at 13,000 g for 3 min to ensure removal of ethanol from the column. One-hundred microliters of H₂O was then added to the column and the DNA was collected by centrifugation at 13,000 g 2 min. Positive transformants were identified by digesting out the ligated DNA fragment using the same restriction enzymes that were used to clone it into the vector (see 2.3.5). Restriction digest reactions were then run on agarose gels (see 2.3.4) to confirm the presence of insert DNA.

2.3.9 Glycerol stocks

Confirmed positive clones were stored permanently by adding 500 µl of 80% glycerol to 1 ml of an overnight culture, mixing them together and then snap freezing the culture in liquid nitrogen and storing it at -80°C.

2.3.10 Fusion protein expression

A scrape from a glycerol stock of positive transformants was streaked onto an LB-agar plate containing ampicillin (50 µg/ml) and allowed to grow overnight at 37°C. Colonies were then picked and grown overnight in 2YTA media (16 g tryptone, 10 g yeast extract and 5 g

NaCl₂/L) containing ampicillin (50 µg/ml) at 37°C with shaking (200 rpm). The following morning, cultures were back diluted 1:100 in 2YTA media, and allowed to grow to the log phase (OD₆₀₀ 0.4-0.5), at which point IPTG was added to a final concentration of 0.1 mM. Cells were allowed to grow at 37°C with shaking for a further 2 h, and were then collected by centrifugation and frozen at -20°C until further use.

2.3.11 Fusion protein preparation

Fusion proteins were extracted from induced *E.coli* following the methods described by Frangioni and Neel (1993). Briefly, cell pellets were resuspended and lysed in a 2% N-laurylsarcosine (sarkosyl) solution containing 5 mM DTT and 5 mM EDTA. Cell debris was then collected by centrifugation at 10,000 rpm for 15 min and Triton-X was added to the supernatant to a final concentration of 1% (v/v). The supernatant containing the fusion proteins was then incubated with glutathione-agarose beads (Sigma) for a minimum of 20 min at 4°C. Agarose beads were collected by centrifugation at 3,000 rpm for 5 min and washed three times with ice cold phosphate buffered saline (PBS) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). Fusion proteins were eluted from the agarose beads with an elution buffer containing 5 mM reduced glutathione, 50mM tris-HCl and 5mM EDTA (pH 9.5) and mixed with equal volumes of 1 x Laemmli sample buffer and subjected to SDS-PAGE.

2.3.12 Gel purification of fusion proteins

12% SDS-PAGE gels were prepared as described (see 2.2.7.8) and approximately 250 µl of the fusion protein/bead solution was loaded and run at 200 V for 40 min. Bands corresponding to the correct size were excised from the gel using a reference lane which had been stained in coomassie blue and destained in 10% acetic acid as a reference. Fusion proteins were eluted from gel slices using Bio-Rad's Electro-Eluter Model 422 according to the manufacturer's instructions. Aliquots of purified fusion proteins were run on SDS-PAGE gels to verify the presence of the correct fusion protein.

2.3.13 Immunisations

Two rabbits (New Zealand white) were injected subcutaneously with approximately 30 µg of the purified GST-calpain-10 fusion protein. For the primary injection, an emulsion was made with 1 ml of the antigen and 1 ml of Freund's complete adjuvant, and was injected over four sites (0.25 ml/site) on each animal. Booster injections were given at 3, 5 and 7 weeks following the primary injection until maximum titre was reached. For the booster injections, the antigen was prepared in the same way, except that the emulsion was made with Freund's incomplete adjuvant. Bleeds were taken prior to the primary injection and before each booster injection to test for the presence of any non-specific cross reacting antibodies. Approximately 3-5 mls of blood was taken from the

ear of each rabbit. Blood was “ringed” to loosen the clot from the tube and was left overnight at 4°C. Serum containing the IgG fraction was collected the next day and stored at -20°C until used.

2.3.14 Calpain-10 antisera from the University of Chicago

Calpain-10 antisera was also obtained as a gift from Prof. G.I.Bell at the University of Chicago, USA for comparison with the internally produced one. This was also a rabbit polyclonal antibody but was raised against a small peptide sequence corresponding to amino acids 35-50 of human calpain-10a (domain I-II, **chapter 1, Fig 1.4**) conjugated to keyhole limpet hemocyanin (KLH) and was designated N7. When obtained, this antibody had not been tested for use in human skeletal muscle extracts and therefore required verification for specificity, as did the calpain-10 antisera raised in-house.

2.3.15 Verification of calpain-10 antisera

2.3.15.1 Dot-blot tests

Dot-blot tests were initially used to test for the presence of cross reacting antibodies at each stage in the immunisation schedule. Serial 10-fold dilutions of the antigen (see 2.3.12) were dotted onto strips of nitrocellulose, which were then blocked with 5% (w/v) Marvel TBS-T for

at least 1 h, and then incubated with serum which was diluted to 1:50, 1:500 and 1:5000 concentrations in 5% (w/v) Marvel TBS-T. The strips were then washed for 30 min with 1% (w/v) Marvel TBS-T and incubated with anti-rabbit IgG alkaline phosphatase secondary antibody for 20 minutes. Strips were again washed for 30 min with 1% (w/v) Marvel TBS-T and the colour reaction was initiated with the addition of the enzyme substrate (Sigma Fast™ 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium tablets).

2.3.15.2 Western blot analysis

Each antibody was tested in the first instance on Western blots containing human skeletal muscle protein extracts and also on Western blots containing whole brain lysates from wild type and calpain-10 knock-out mice, which were produced in the laboratory of Prof. G.I.Bell, University of Chicago. In the case of the internally produced antibody, the Western blot included a lane which contained the fusion protein that was used to immunise the animals to confirm the presence of specific cross-reacting antibodies. In addition, for the internally produced antibody, the final bleed serum was compared to the pre-immune serum in terms of specific cross reactivity.

2.3.15.3 In vitro transcription and translation of human calpain-10

To investigate the specificity of the N7 antibody to the full length human calpain-10 protein, the full length protein was produced *in vitro* in a relatively pure form. The entire calpain-10 coding sequence, or open reading frame (ORF) in the pcDNA-3.1-HisA vector (Invitrogen) was first obtained from Prof. G.I.Bell and this was subcloned into the TNT vector (Promega). To achieve this, the calpain-10 ORF was amplified from the pcDNA-3.1-HisA vector using the forward primer 5'-AAAGAATTCGAGGCAACCGGCTGCAGATG-3' and the reverse primer 5'-AAAGCGGCCGCTGCAAATCATCAGCGCTCAT-3' in a 20 µl final volume containing 10 pmol each primer, 1.5 mM MgCl₂, 10 µM each dNTP, 1 x reaction buffer, 0.25 U Taq Gold and ~ 30 ng template DNA. PCR cycling conditions were 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes and a final extension step of 72°C for 10 minutes. The PCR fragment and TNT vector (Promega) were digested with EcoRI and NotI and run on an agarose gel using the standard techniques described above (2.3.5 and 2.3.4). Both products were then purified with an ethanol precipitation (see 2.3.3) and the calpain-10 ORF was then ligated into the TNT vector using the EcoRI and NotI sites in the vector (see 2.3.6).

The pcDNA-3.1-HisA-calpain-10 DNA vector was then transcribed and translated in a 25 µl reaction using the rabbit TNT Coupled Reticulocyte

Lysate Transcription/Translation System (Promega) according to the manufacturers instructions. Briefly, 0.5 µg of the DNA was mixed with 12.5 µl reticulocyte lysates, 1 µl TNT buffer, 0.5 µl amino acid mix (-methionine), 1 µl [³⁵S] methionine (>1000 Ci/mM), 0.5 µl T7 RNA polymerase, 0.5 µl RNasin and 8 µl of H₂O. Reactions were run in duplicate in the presence or absence of [³⁵S] methionine and were run on 10% (w/v) SDS-PAGE gels and transferred to PVDF membranes as described above (2.2.7.9). Confirmation of the expression of the full length protein was initially determined by the detection of the [³⁵S] labelled protein. For this the PVDF membrane was dried and placed into a western blot cassette and a phosphoscreen (Kodak, Rochester, USA) was then placed on top of the membrane and left in place overnight, after which the phosphoscreen was scanned in a phosphorimager (Bio-Rad). Specificity of the N7 antibody to the *in vitro* produced protein was then investigated by immunoprobng the other PVDF membrane containing the unlabelled protein with each antibody (see 2.2.7.10).

2.4 Calpain-3 antisera

The calpain-3 antisera used throughout this thesis was produced internally by Dr. T. Parr of the Department of Nutritional Sciences, University of Nottingham and has been described in detail previously (Parr et al., 1999).

2.5 Determination of calpain-10 SNPs

Each individual's genotype at SNP-43, Indel-19 and SNP-63 were determined essentially as described by Carlsson et al (2004) for SNP-43 and by Evans et al. (2001) for Indel-19 and -63, with minor modifications.

SNP-43 was amplified using the forward primer 5'-GCTGGCTGGTGACATCAGTGC-3' and reverse primer 5'-ACCAAGTCAAGGCTTAGCCTCACCTTCATA-3' in 20 µl volumes containing 10 pmol each primer, 1.5 mM MgCl₂, 10 µM each dNTP, 1 x reaction buffer, 0.25 U Ampli Taq Gold and ~ 25 ng human genomic DNA. PCR cycling conditions were 94°C for 5 minutes followed by 32 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final extension step of 72°C for 10 minutes. PCR products were digested for 16 h at 37°C with 10 U NdeI in 40 µl reactions and run on 4% agarose gels. If the individual was homozygous for the G allele (allele 1) then a single band of 254 bp was visualised on the gel and if they were homozygous for the A allele (allele 2), then two bands of 223 and 31 bp were seen. A G/A heterozygote was identified by the presence of all three bands.

Indel-19 was amplified using forward primer 5'-GTTTGGTTCTCTTCAGCGTGGAG-3' and reverse primer 5'-CATGAACCCTGGCAGGGTCTAAG-3' using the same reaction

conditions as above except that the annealing temperature was 60°C. PCR products were run on 3% (w/v) agarose gels; allele 1 (two repeats) was visualised as 155 bp and allele 2 (three repeats) as a band of 187 bp.

SNP-63 was amplified using the forward primer 5'-AAGGGGGGCCAGGGCCTGACGGGGGTGGCG-3' and reverse primer 5'-AGCACTCCCAGCTCCTGATC-3'. The PCR conditions were the same as those for Indel-19, except that the annealing temperature was 62°C. PCR products were digested with 2 U of HhaI in 1 × NE4 buffer at 37°C for 2 h. The digested products were separated on 3% (w/v) agarose gels. Alleles 1 (C) and 2 (T) were seen as 162 and 192 bp products, respectively.

2.6 Statistics

Data in all chapters were analysed using a general linear model (GLM) for repeated measures (SPSS, Version 13.0). In **chapter 3** this was a one-way analysis (time) and in **chapters 4, 5 and 6** this was a two way analysis (treatment and time). Student's t test (two-tailed) was used to compare paired data where appropriate, with Bonferroni corrections if required (i.e. for multiple t-tests) to avoid type 1 error. When missing data was evident, and when indicated, a linear mixed model with repeated measures was used to eliminate the effects of loss of data and power. In a standard GLM repeated measures analysis, when data is

missing at any time point for a subject, all data for that subject is automatically removed (a so-called “listwise deletion”) and in some cases this can result in significant loss of statistical power leading to an increased risk of type 2 error. This was a particular problem for the analysis of mRNA and protein expression in **chapter 4** and **chapter 5**, where occasionally protein and/or total RNA was not extracted in some subjects due to small muscle biopsy samples at certain time points leading to random missing data points. A linear mixed model is able to analyse data with missing values and is therefore an improvement on the GLM, particularly in cases where a small number of missing values are present in a repeated experimental design. The effects of SNPs in chapter 7 was analysed using paired t tests for independent samples. A P value less than 0.05 was considered significant. All data are expressed as mean (\pm SEM) throughout this thesis and this includes applies to all text, figures and tables.

3 Short-term fasting and skeletal muscle calpain expression in healthy humans

3.1 Introduction

The adaptive response to fasting involves a series of metabolic alterations, characterised initially by increased release of free fatty acid (FFA) from endogenous stores and reduced production and oxidation of glucose. Whilst the exact mechanisms underlying these adaptations are not fully understood, the physiological responses to starvation are commonly accompanied by a significant reduction in whole body insulin sensitivity (Mansell and Macdonald, 1990) and alterations in the expression of many genes that are important for glucose oxidation and transport. The reduction in glucose uptake with fasting may be mediated by changes in the expression of the glucose transporter GLUT4 (see 1.2.1.4). In fasted rats, levels of GLUT4 mRNA in adipose tissue are reduced with fasting and refeeding the rats leads to a complete recovery of the GLUT4 mRNA (Sivitz et al., 1989; Berger et al., 1989; Camps et al., 1992). In skeletal muscle the response of GLUT4 to starvation is not as clear with some studies demonstrating an increase (Woloshak et al., 1993; Neuffer et al., 1993) and others no change (Kraegen et al., 1993) in GLUT4 content following fasting in rodents. Therefore, the role of GLUT4 in fasting mediated insulin resistance in humans remains unclear. This is especially true given that

GLUT4 mRNA and protein levels are not different in the skeletal muscle of type 2 diabetic patients or insulin resistant obese subjects (Pederson et al., 1990; Shepherd and Kahn, 1999). In these cases, it is likely that changes in GLUT4 translocation and not expression mediate, in part, the insulin resistance seen in these subjects.

Calpain-10 has been linked to GLUT4 mediated glucose uptake in adipocytes *in vitro* (see 1.9.2.2). In rat adipocytes, specific inhibition of calpain-10 using antisense technology reduced insulin mediated glucose transport by 50% and significantly reduced GLUT4 translocation to the plasma membrane via effects on actin reorganisation (Paul et al., 2003). However, as outlined in chapter 1, there is currently no direct evidence linking calpain-10 and GLUT4 mediated pathways of glucose uptake in skeletal muscle. The muscle specific calpain-3 has previously been shown to be affected by fasting treatment in lambs (Ilian et al., 2001). In this study, skeletal muscle calpain-3 protein expression was significantly elevated after only one day of fasting but after seven days was lower than pre-fast values (Ilian et al., 2001). Moreover, fasting and refeeding chicks led to marked increases and decreases in skeletal muscle calpain-3 mRNA, respectively (Nakashima et al., 2005).

Experiments primarily carried out in rodents suggest that transcriptional regulation of a number of key genes involved in lipid and CHO metabolism may also be responsible for the metabolic adaptations to

starvation and refeeding. Short-term fasting in rats and mice has been previously shown to increase the expression of genes involved in fatty acid transport and beta oxidation such as lipoprotein lipase (LPL), carnitine palmitoyltransferase I (CPT1), long chain acyl CoA dehydrogenase (LCAD), the fatty acid transporter CD36 and uncoupling protein 3 (UCP3) (Holst et al. 2003; de Lange et al. 2004), indicating that increased fatty acid influx into muscle during starvation is accompanied by an adaptive increase in gene expression. Regulation of oxidative glucose disposal appears to be controlled at the transcriptional and post-transcriptional level. Suppression of skeletal muscle pyruvate PDC activity plays a major role in the downregulation of CHO oxidation in response to starvation (Sugden et al. 1993). In humans, starvation was shown to increase PDK4 transcription (Pilegaard et al. 2003a) and mRNA content in skeletal muscle (Pilegaard et al. 2003a; Spriet et al. 2004).

The aim of the present study was to investigate the effects of fasting induced insulin resistance on the mRNA and protein expression of calpain-10, -3 and GLUT4 in human skeletal muscle. It has been shown that a reduction in calpain-10 is associated with insulin resistance in humans and it was hypothesised that fasting induced insulin resistance would be linked to a similar reduction in calpain-10 mRNA and protein expression. Based on the limited available evidence, this reduction in calpain-10 would be expected to lead to a reduction in GLUT4 translocation and, if GLUT4 is a substrate for

calpain-10, possibly an accumulation of GLUT4 content. It was hypothesised that a restoration of insulin sensitivity with high carbohydrate refeeding would reverse these changes in calpain-10 and GLUT4 levels. On the other hand, calpain-3 was expected to increase with fasting as has been previously described in animal models.

3.2 Subjects and Methods

3.2.1 Subjects and experimental design

Ten healthy male volunteers (age 26 ± 1 yr, body mass 81 ± 4 kg, BMI 26 ± 1 kg.m²) participated in this study, which was approved by the University of Nottingham Medical School Ethics Committee. All subjects underwent a 48 h period of fasting followed by a 24 h period of refeeding with a high carbohydrate diet (total energy 3086 ± 19 kcal of which 75% energy as CHO, 10% fat and 15% protein). During fasting, subjects refrained absolutely from food but were allowed water, electrolytes, non-sugared carbonated drinks and, except on study days, black non-sugared decaffeinated coffee and tea. Subjects consumed 80 mmol of sodium and 40 mmol of potassium daily as slow release tablets to minimize the potentially confounding effects of fluid deprivation and intravascular volume depletion on cardiovascular reflexes and sympathetic nervous system activity. Subjects were required to continue with their normal daily activities but to refrain from formal heavy exercise sessions during the period of starvation. The high CHO diet was designed after analysing the subjects diet with Microdiet software (Downlee Systems Limited, UK) using dietary figures produced by the subjects who were requested to weigh and record their normal food intake for three days prior to the trial. On three occasions, before and after 48 h of starvation and after 24 h of refeeding, subjects underwent a 16 min insulin tolerance test (ITT) to quantify insulin

sensitivity (3.2.2). On day 1 of the study, prior to the commencement of fasting and on day 4 following refeeding, subjects consumed a standardized breakfast (providing 1g CHO/kg body mass) 4 h before they arrived at the laboratory, at which point baseline measurements were taken. Muscle biopsy samples were obtained from the vastus lateralis before and after 24 h and 48 h of starvation, and after 24 h of refeeding and snap frozen in liquid nitrogen until further use.

3.2.2 Insulin tolerance test (ITT)

In this chapter, whole body insulin sensitivity was estimated using an insulin tolerance test (ITT) (Bonora et al., 1989). Briefly, at 0 min of the ITT, an intravenous bolus of human Actrapid insulin (0.1 U per kg body mass) was administered and blood samples for glucose determination were then taken every 2 min. The test was terminated at 16 min and a 25 ml bolus of 20% glucose was infused to prevent symptoms of hypoglycaemia. Following this, a high CHO meal was provided. Blood glucose concentrations were checked for stability before the subject was allowed to leave the laboratory. Data from this test were expressed as the percentage decline in blood glucose (mM) per min from 4 to 16 min after the insulin injection, since no changes in blood glucose concentration were observed within the first 4 min of the ITT test (Bonora et al., 1989). The ITT has been used widely as an index of insulin sensitivity and the results from this test have been shown in direct comparison studies to correlate closely (r between 0.81-0.86) with

results from the hyperinsulinaemic-euglycaemic clamp technique (Akinmokun et al., 1992; Bonora et al., 1989).

3.2.3 Blood analysis

Blood glucose, serum insulin and plasma FFA were analysed as described (**general methods, 2.2.2**).

3.2.4 RNA extraction and Real Time PCR

Total RNA was extracted from skeletal muscle biopsy samples and qRT-PCR was performed on reverse transcribed cDNA as described (**general methods, 2.2.7.4**). The effects of fasting and refeeding on the levels of calpain-10 and -3 and GLUT4 mRNA were assessed. The real-time PCR primers and probes used are listed in **appendix 2**.

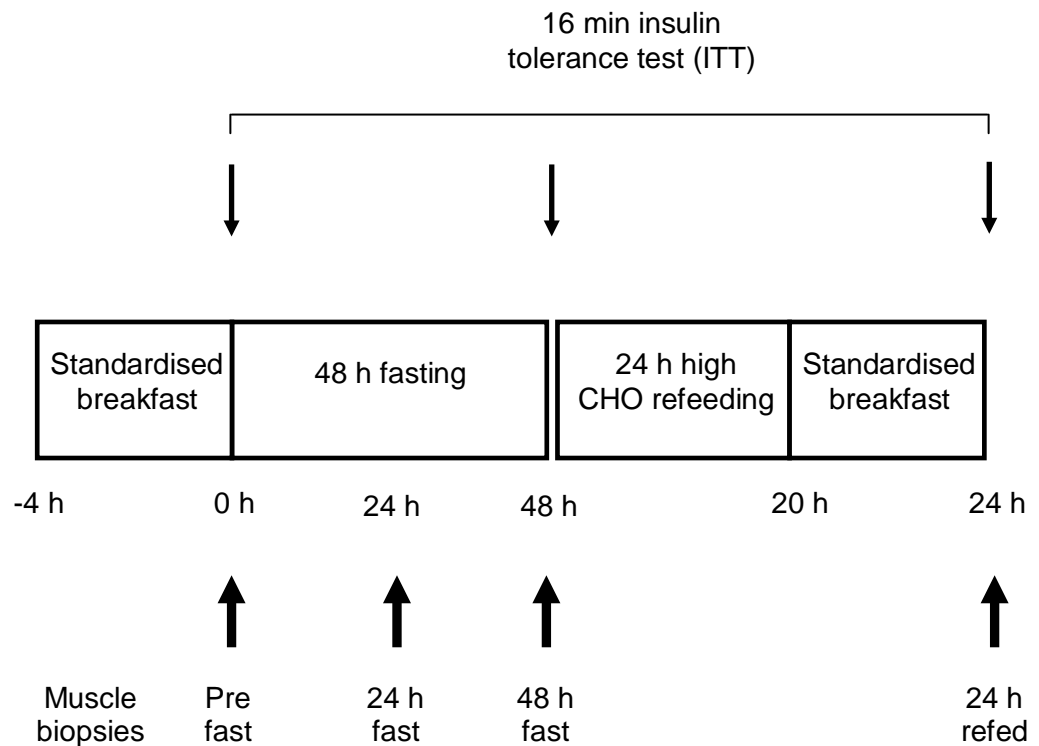


Figure 3.1 Schematic diagram of the starvation study protocol.

Subjects reported to the lab following a standardised breakfast and began 48 h of fasting. Following fasting, subjects then underwent 24 h of high CHO refeeding. Insulin tolerance tests were performed before and after 48 h of fasting and after 24 h of refeeding. Blood samples and muscle biopsies were taken before, during and after fasting and after refeeding for the measurement of blood metabolites and mRNA and protein expression, respectively.

3.2.5 Calpain antisera

Calpain-10 and -3 antisera were produced and obtained as described in the **general methods (2.3 and 2.4)**.

3.2.6 Western blot analysis of Skeletal Muscle Protein Extracts

Calpain-10 and -3 protein levels were quantified in the skeletal muscle protein pellets as described in the **general methods (2.2.7.10)**. For the quantification of calpain-3 autolysis products in this chapter, autoradiograph films were exposed for longer to allow for more accurate quantification and were then normalised to the same desmin values. Protein levels of GLUT4 were determined for two subjects only due to limited tissue. For this, 30 µg of soluble muscle protein extract on nitrocellulose membrane was probed with an anti-GLUT4 antibody, diluted 1:500 (Santa Cruz Biotechnology, USA) using the same procedure as for calpain-3 (**general methods, 2.2.7.10**).

3.2.7 Statistical analysis

Statistical analysis was performed as described in the **general methods (2.6)**.

3.3 Results

3.3.1 Calpain-10 antisera

In this chapter, the determination of calpain-10 protein expression was performed for the first time in this thesis and therefore the characterisation of each calpain-10 antibody is presented here. Determining the specificity of the antibodies was important for the future quantification of calpain-10 protein levels in this and in the subsequent chapters of this thesis.

3.3.1.1 Recombinant calpain-10 purification

In Fig 1A, it is shown that the GST-calpain-10 fusion protein was expressed at high levels in *E.Coli* and contributed to a significant proportion of the total protein content of the induced *E.Coli*. It is also shown that the expression of the fusion protein was much higher in the induced *E.Coli* when compared to the non-induced cells (Fig 1A). This expressed fusion protein was purified successfully (Fig 3.2B) and rabbits were immunised with the purified protein as described in the **general methods (2.3.13)** section.

3.3.1.2 Antisera verification

Initial investigations into the internally produced antisera showed that one of the antibodies (designated 690) cross reacted strongly with the antigen on dot-blot tests and Western blots (Fig 3.3A and 3.3B) and was able to detect a band of approximately 73 kDa on Western blots of human skeletal muscle extracts (Fig 3.3C). However, the pre-immune serum was also able to detect a very light band at approximately the same size suggesting that this band may be non-specific (Fig 3.3C). Further studies showed that this antibody was not able to distinguish between wild type and calpain-10 KO mice on Western blots of whole brain lysates (Fig 3.3D).

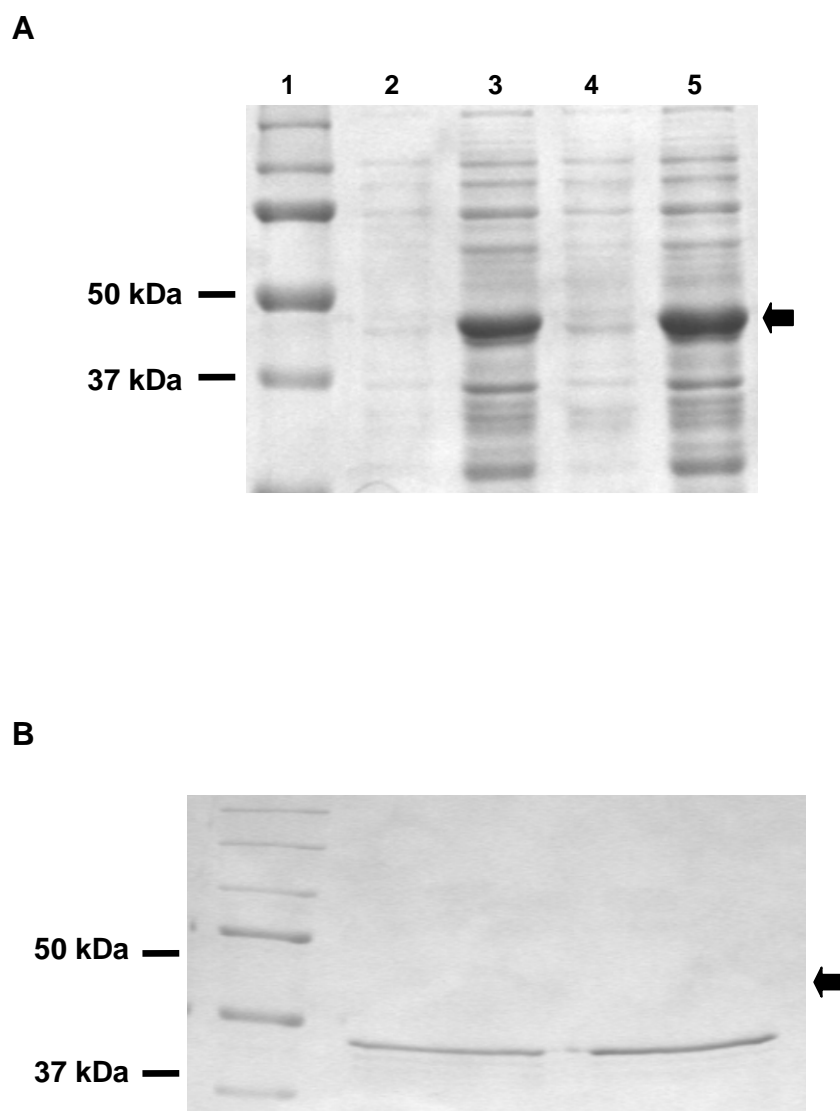


Figure 3.2 Production (A) and purification (B) of a recombinant GST-calpain-10 fusion protein.

In the upper panel (A), lane 1 on the SDS-PAGE gel contains the molecular weight marker, lanes 2 and 4 the uninduced *E.coli* lysates and lanes 3 and 6 contain lysates from *E.Coli* that were induced to express the recombinant GST-calpain-10 fusion protein, indicated by the arrow. The gel purified protein, shown on a separate SDS-PAGE gel (B) was used for subsequent immunisations.

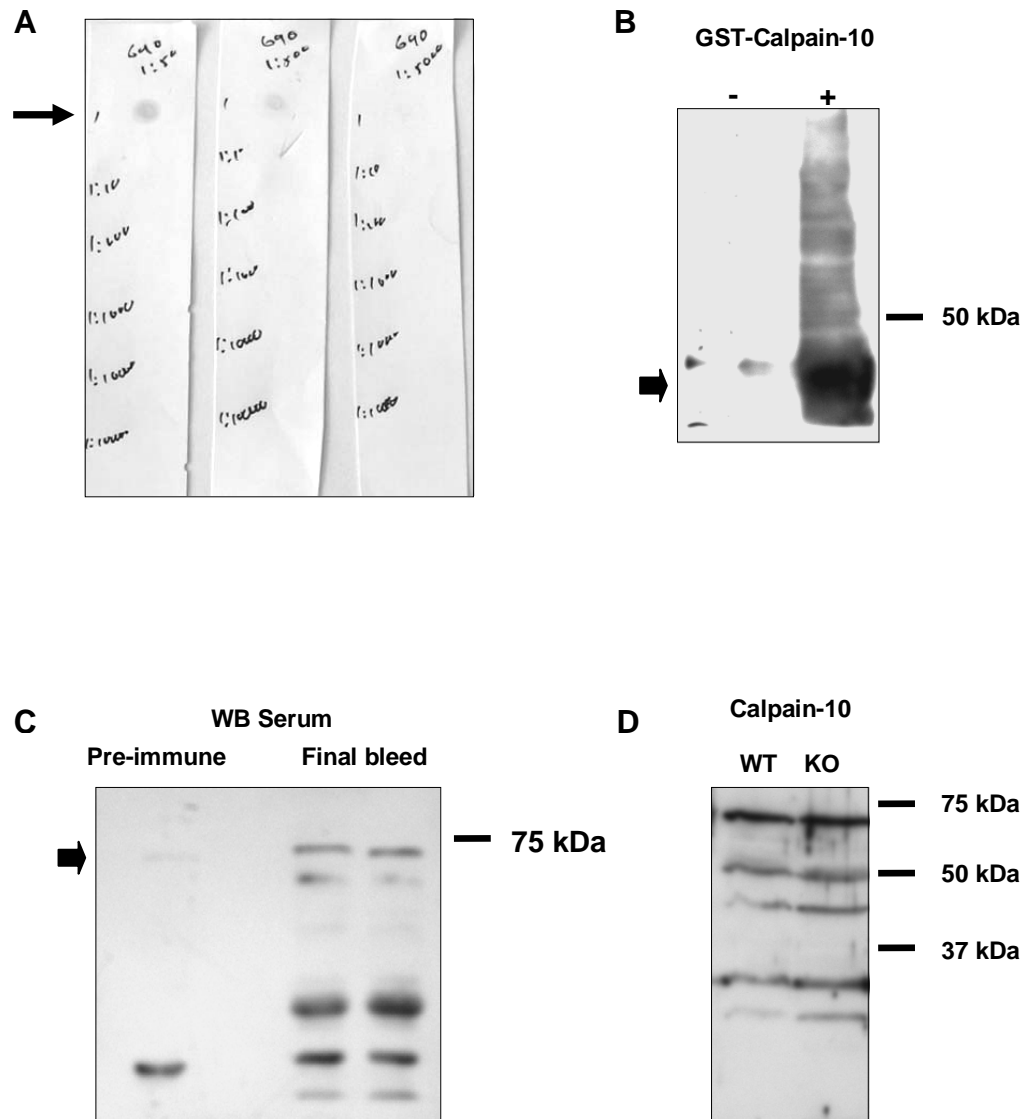


Figure 3.3 Characterisation of the polyclonal antibody (609) raised against a recombinant GST-calpain-10 protein.

Testing of the 609 antibody on dot-blot tests containing the purified antigen (A) and against recombinant *E.Coli* induced to express the same antigen (B) revealed specificity for the 46 kDa GST-calpain-10 protein. Subsequent testing on Western blots, however, revealed that the 73 kDa band recognised by the 609 serum in human skeletal muscle was also detected by the pre-immune serum from the same animal, indicated by the arrow (C). Finally, the antibody did not differentiate between calpain-10 WT and KO mice on Western blots of whole brain lysates (D).

In contrast, Western blot analysis of insoluble human skeletal muscle protein with the antibody obtained from the University of Chicago (designated N7) revealed a major band for calpain-10 at 75 kDa, but also demonstrated the presence of smaller bands at 60 kDa and 32 kDa (Fig 3.4A). The N7 antibody also clearly recognised a 75 kDa *in vitro* translated protein, corresponding to full length human calpain-10 (isoform a) (Fig 3.4B) and detected 32 and 25 kDa proteins in whole brain lysates from wild type, but not calpain-10 knock-out mice (Fig 3.4C),

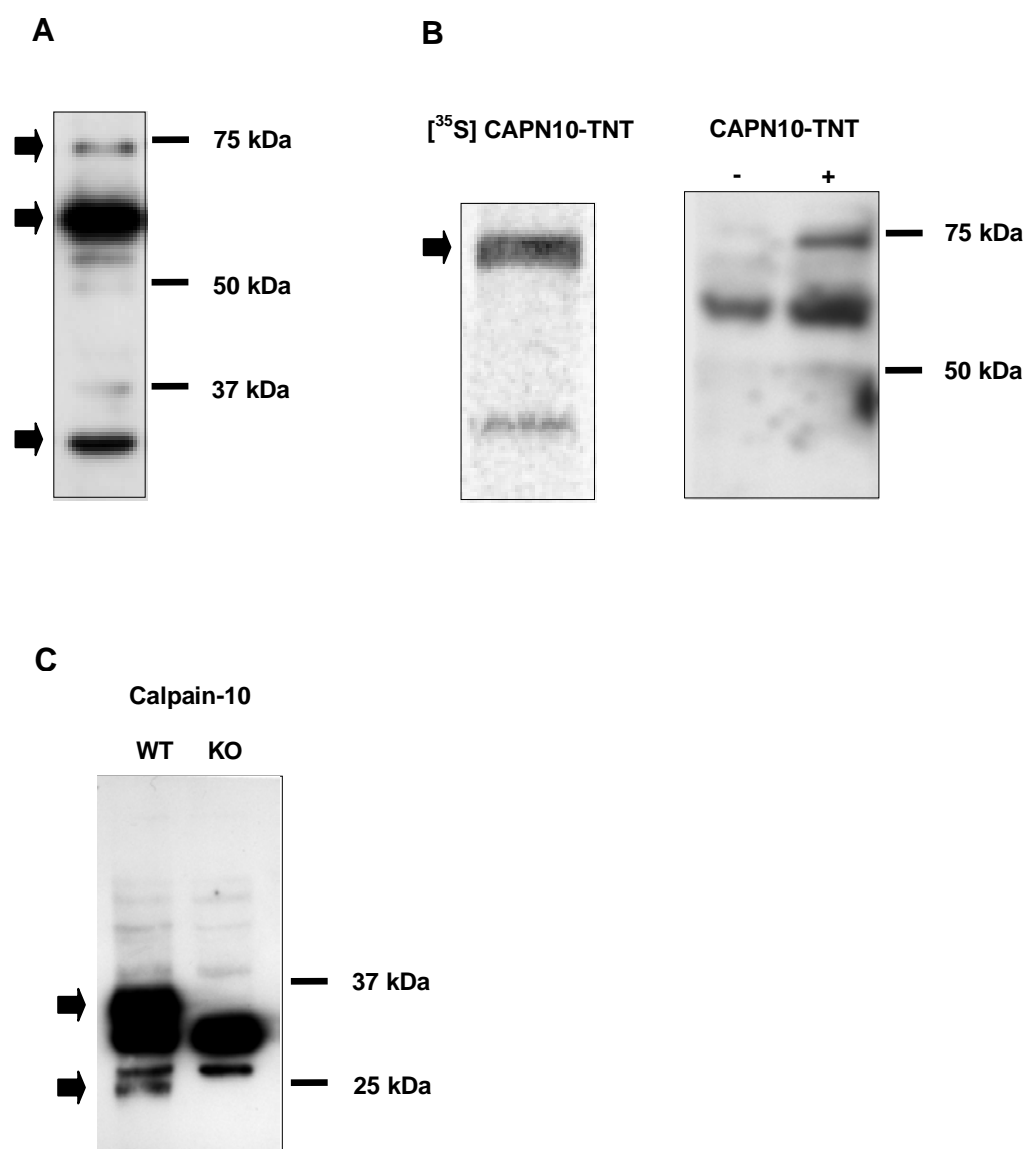


Figure 3.4 Characterisation of the N-terminal antibody (N7) obtained from the University of Chicago.

Western blot analysis of insoluble human skeletal muscle protein with the N7 antibody revealed major bands for calpain-10 at 75, 60 and 32 kDa, as shown by the arrows (A). The N7 antibody also clearly recognised a 75 kDa *in vitro* translated protein (B) and detected 32 and 25 kDa proteins on Western blots of whole brain lysates from wild type, but not calpain-10 knock-out mice (C). The solid black bars represent the molecular weight markers.

3.3.2 Insulin sensitivity and blood measurements

The ITT used in the present study measures the decrease in blood glucose concentration in response to an intravenous administration of insulin and thus provides an index of whole body insulin sensitivity. As expected, 48 h fasting significantly reduced the percentage decline in blood glucose per minute during the ITT by ~45% when compared to pre fast values (PRE FAST 5.16 ± 0.44 vs. 48 h FAST 2.94 ± 0.31 %/min, $p < 0.01$) and this partially recovered to basal values following refeeding (PRE FAST 5.16 ± 0.44 vs. REFED 4.02 ± 0.34 %/min, $P < 0.05$) (Fig 3.5A). This reduction in the fall in blood glucose concentration during the ITT at 48 h post fasting coincided with a decrease in fasting blood glucose concentrations (PRE FAST 4.3 ± 0.1 vs. 48 h FAST 3.5 ± 0.1 mmol/L, $P < 0.001$) and a reduction in fasting serum insulin concentrations (PRE FAST 29.5 ± 3.5 vs. 48 h FAST 16.4 ± 1.2 pmol/L, $P < 0.01$) with both fully recovering to basal values following refeeding (Table 3.1). In contrast, plasma FFA markedly increased with fasting more than 2-fold from its baseline value ($P < 0.001$) (Table 3.1).

Table 3.1 Blood glucose, serum insulin and plasma FFA before and after fasting and refeeding (n = 10).

	Pre fast	24 h fast	48 h fast	24 h refed
Blood Glucose (mmol/l)	4.3 ± 0.1	4.09 ± 0.1	3.51 ± 0.1***	4.44 ± 0.1
Serum Insulin (mU/l)	4.9 ± 0.6	4.4 ± 0.5	2.7 ± 0.2**	5.3 ± 0.9
Plasma FFA (mmol/l)	0.46 ± 0.07	0.76 ± 0.12	0.97 ± 0.10***	0.45 ± 0.08

P<0.01, *P<0.001 vs. pre fast values

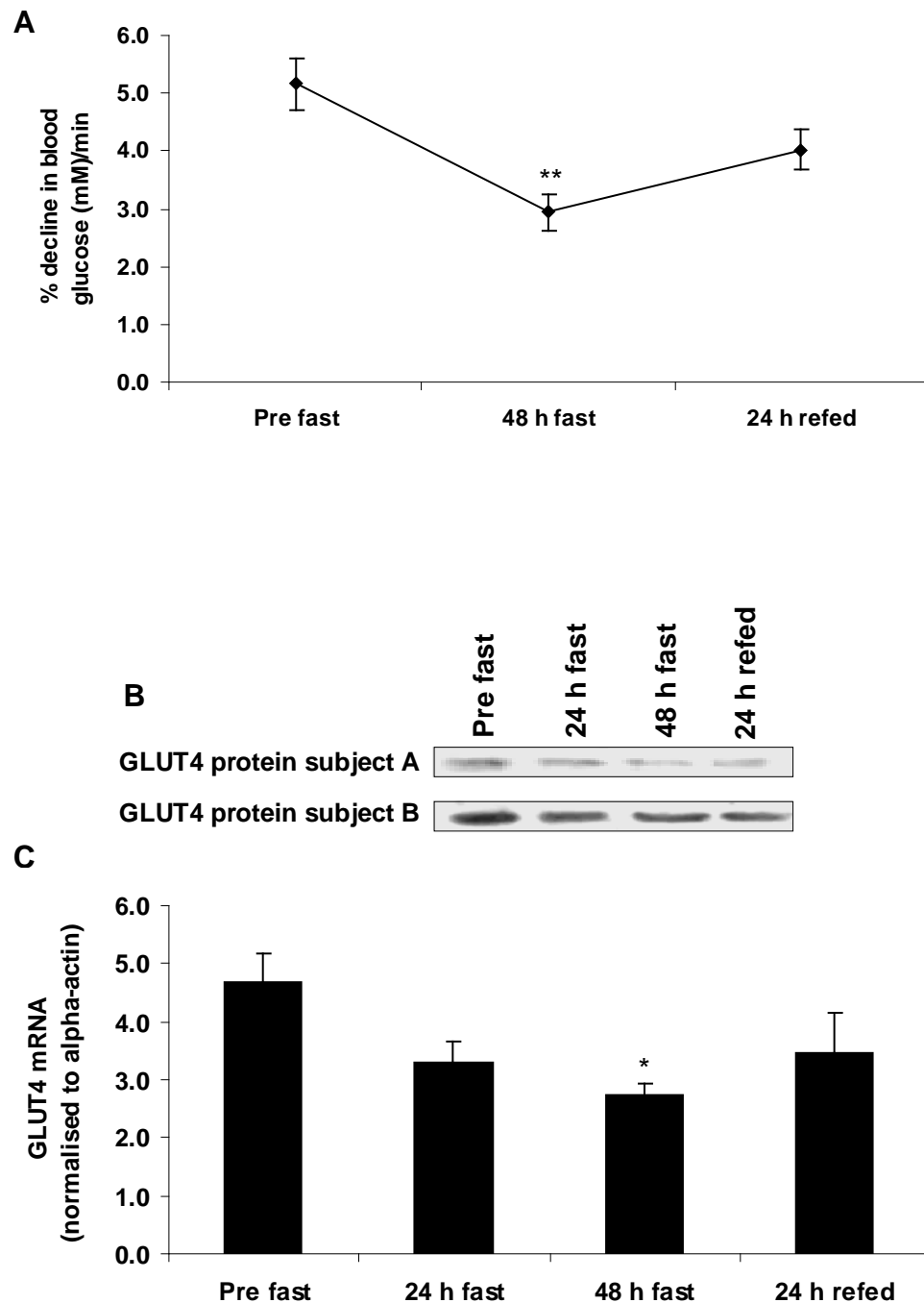


Figure 3.5 The effect of fasting and refeeding on whole body insulin sensitivity ($n = 10$) (A) and on skeletal muscle GLUT4 protein ($n = 2$) (B) and mRNA ($n = 10$) (C) expression.

Insulin sensitivity is expressed as the percentage decline in blood glucose (mM) per min and was determined using an ITT. Also shown is Western blots for GLUT4 protein performed on two subjects (B). * $P < 0.05$, ** $P < 0.01$ vs. pre fast values.

3.3.3 Calpain-10 and GLUT4 expression

Basal GLUT4 mRNA levels were gradually reduced following 24 h and 48 h of fasting and failed to recover fully to basal values with refeeding (PRE FAST 4.7 ± 0.5 vs. 24 h FAST 3.3 ± 0.4 vs. 48 h FAST 2.8 ± 0.2 vs. REFED 3.5 ± 0.7 , $P < 0.05$) (fig 3.5C). Post-hoc analysis revealed that GLUT4 mRNA was reduced significantly after 48 h fasting ($P = 0.013$), but not 24 h, and was not significantly different to pre fast following refeeding (Fig 3.5C). Due to limited tissue, the determination of GLUT4 protein content was only possible in two subjects in the present study; GLUT4 protein levels in these two individuals appeared to match the changes in GLUT4 mRNA and were also reduced with fasting (Fig 3.5B).

Total calpain-10 mRNA expression remained unchanged by fasting and refeeding (PRE FAST 3.9 ± 0.5 vs. 48 h FAST 3.2 ± 0.3 vs. REFED 4.2 ± 0.9 , Fig 3.6A). As the probe and primers hybridized to an area common to multiple isoforms of calpain-10 mRNA, these data represent the summative expression of five potential calpain-10 mRNA transcripts (10a to 10e). It is likely that isoform 10a represents the majority of the mRNA in skeletal muscle as has been previously demonstrated (Yang et al., 2001). The expression of the 75 kDa protein band was unchanged by fasting and refeeding relative to desmin (Fig 3.6B), as was the expression of the smaller 60 and 32 kDa bands, which may represent various isoforms of calpain-10.

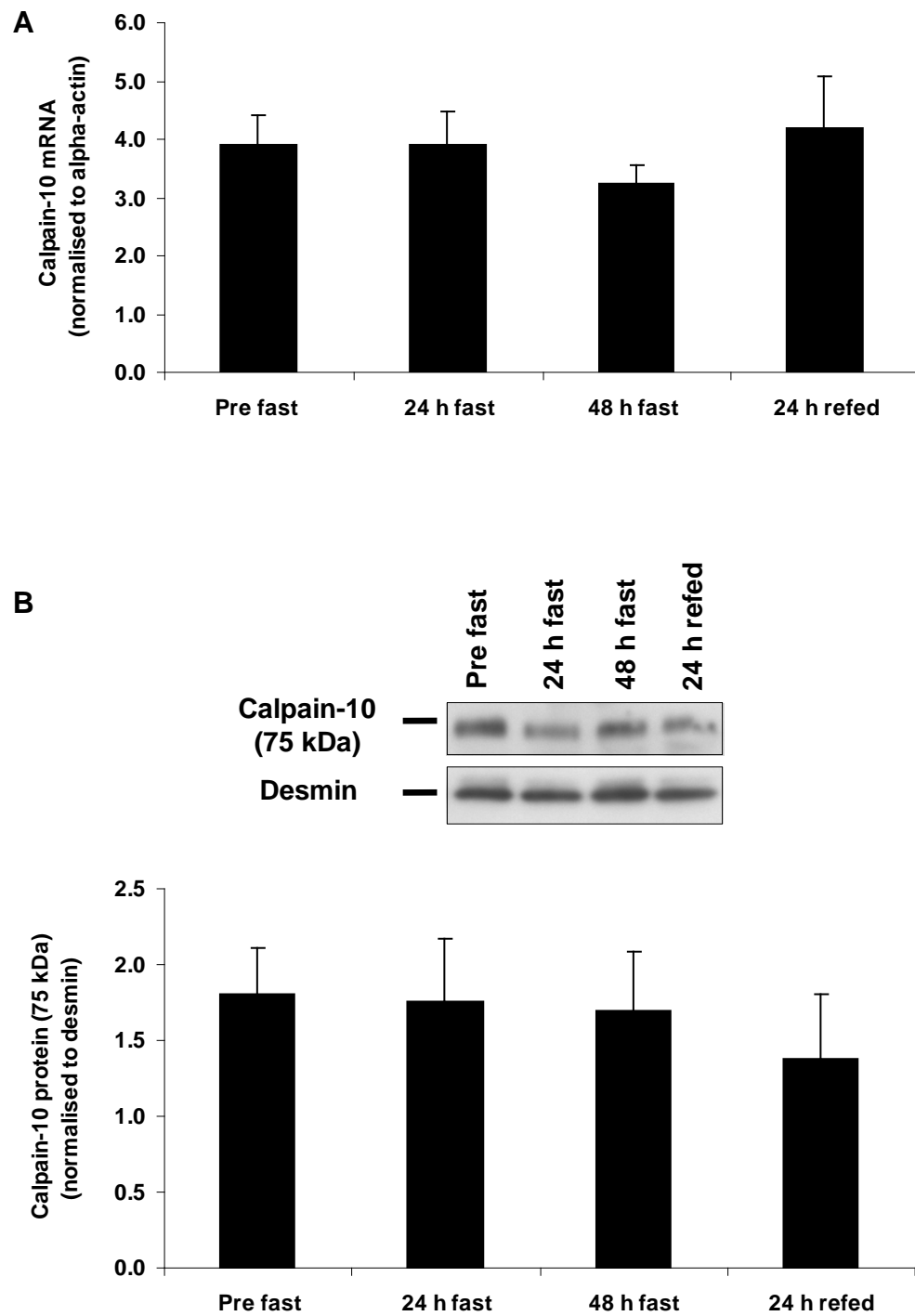


Figure 3.6 Effects of fasting and refeeding on calpain-10 mRNA (A) (n = 10) and full length protein expression (B) (n = 9).

Representative Western blots for calpain-10 (75 kDa) and desmin (55 kDa) are shown (B). Calpain-10 and desmin were measured on the same Western blot.

3.3.4 Calpain-3 expression

Calpain-3 (PRE FAST 2.4 ± 0.2 vs. 48 h FAST 2.3 ± 0.1 vs. REFED 2.5 ± 0.3 , Fig 3.7A) mRNA expression was unaffected by fasting and/or refeeding. Western blot analysis of calpain-3 revealed a major band at ~94kDa corresponding to the full length protein, and some smaller well documented autolysis products at ~60, 58 and 55 kDa (Kinbara et al., 1998) (Fig 3.7B). There was no significant effect of fasting and refeeding on the expression of the full length calpain-3 protein or on the accumulation of these calpain-3 autolysis products (Fig 3.7B).

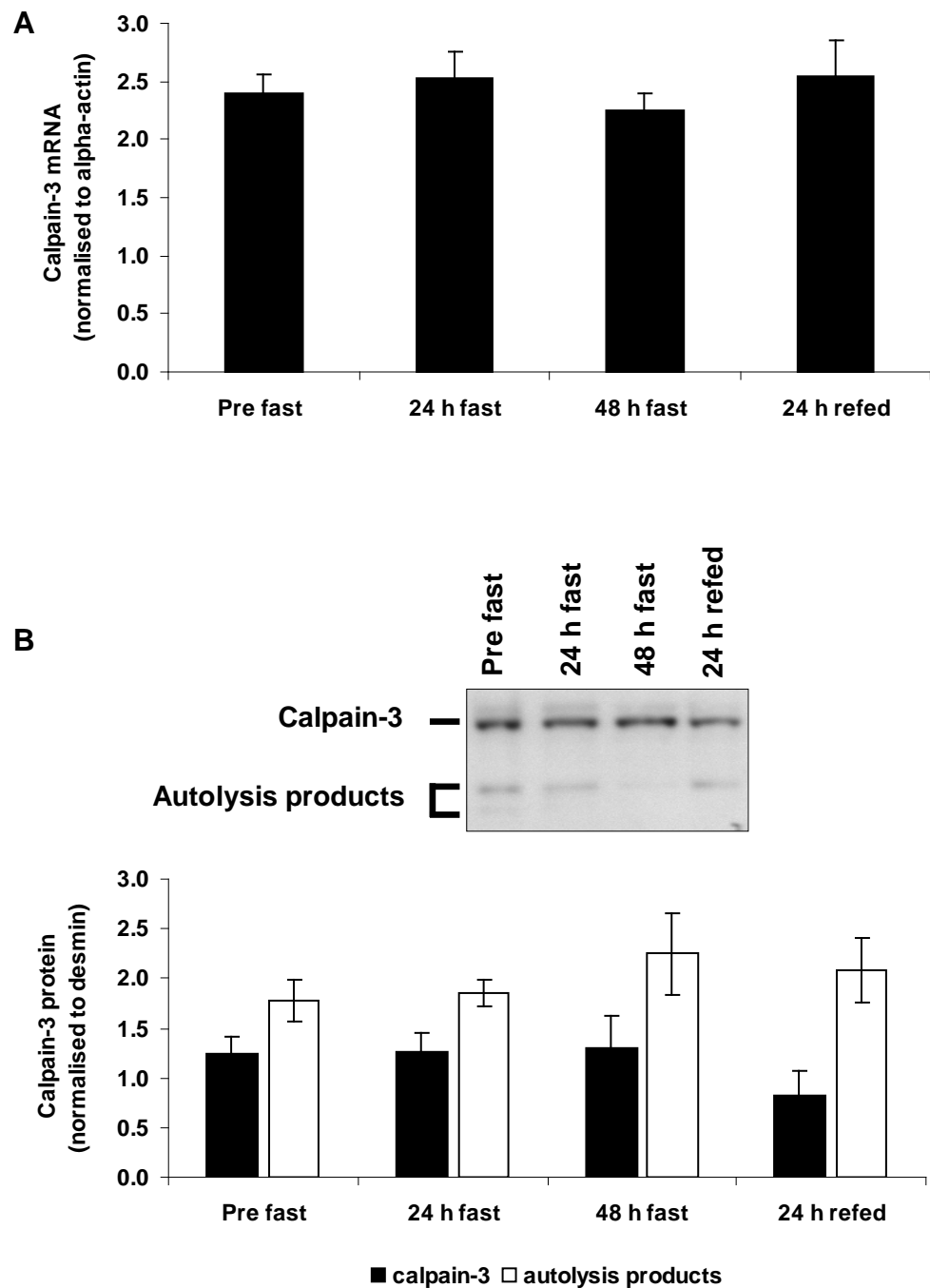


Figure 3.7 Effects of fasting and refeeding on calpain-3 mRNA (n = 10) (A) and protein expression (n = 9) (B).

A representative Western blot of calpain-3 (94 kDa) is shown (B). The quantification of autolysis products (55 – 60 kDa) was performed on different autoradiograph films and it is not intended that the absolute quantity of the autolysis products is compared directly to the quantity of the full length protein, as described in the text (3.2.6).

3.4 Discussion

Previous studies on humans using insulin clamps and stable isotopes have demonstrated a reduction in insulin sensitivity and a shift in basal and insulin-stimulated substrate utilisation from CHO to fat with fasting (Mansell & Macdonald, 1990; Webber et al. 1994). In the basal state after a 48 h fast, forearm muscle demonstrated a reduced glucose uptake and markedly increased glycerol output, indicating increased fat utilisation (Mansell & Macdonald, 1990). During insulin infusion there was marked reduction in glucose uptake by the forearm muscle, indicating skeletal muscle insulin resistance (Mansell & Macdonald, 1990). However, the molecular mechanisms underlying the effects of starvation on insulin resistance were not examined and remain unclear. In the present study, it was hypothesised that the adaptive changes to fasting in skeletal muscle were associated with a reduction in calpain-10 and -3 mRNA and protein expression and possibly an accumulation of GLUT4 content.

The detection of calpain-10 protein in human skeletal muscle is characterised in this chapter for the first time. It was necessary to attempt to produce a human calpain-10 antibody as at the time of production, there were no commercially available antibodies for purchase and the only antibodies used in the literature were designed to cross react with rat calpain-10. The first antibody was produced internally (designated 609) and the other antibody (designated N7) was

obtained as a gift from Prof. G.I.Bell from the University of Chicago, USA. Initial testing of 609 showed that this antibody reacted very strongly with the antigen that was used for immunisations (i.e. the recombinant GST-calpain-10 protein), both on dot-blot tests and on western blots of *E.coli* that were induced to synthesise the recombinant protein in large quantities. However, more detailed analysis showed that this antibody detected a band of approximately 73 kDa in human skeletal muscle. Not only was this band smaller than the predicted size of full length calpain-10 (75 kDa) but it was also visible when probing with the pre-immune serum which was obtained prior to immunization with the recombinant protein. Moreover, 609 failed to distinguish between calpain-10 WT and KO mice on western blots and detected a band of 73 kDa in both WT and KO tissue. In contrast, the N7 antibody detected 32 and 25 kDa bands in WT but not calpain-10 KO mice and also recognised a number of bands on Western blots of human skeletal muscle extracts, one of which corresponded to the predicted size of full length human calpain-10. Finally it was shown that this antibody was able to detect a 75 kDa *in vitro* produced human calpain-10 protein. Based on this evidence, the N7 antibody was deemed the more suitable for use in this chapter and for the remainder of this thesis.

In the present study, fasting reduced insulin sensitivity (as estimated using an ITT) by ~45% and this coincided with a reduction in GLUT4 mRNA and protein levels. GLUT4 mRNA has been shown to be reduced in insulin sensitive tissues following fasting in rats (Sivitz et al.,

1989), and similarly the present study shows for the first time in humans that GLUT4 mRNA is reduced in skeletal muscle following 48 h of fasting and only partly recovers following high carbohydrate refeeding. The changes in GLUT4 expression closely mirrored the alterations in insulin sensitivity with fasting and refeeding, and may account, at least in part, for the failure of insulin sensitivity to fully recover following refeeding. These data are in contrast to studies in humans demonstrating that GLUT4 expression is not correlated with rates of glucose uptake and is not different in the skeletal muscle of insulin resistant and type 2 diabetic patients (Pedersen et al., 1990). Further studies have provided evidence to suggest that GLUT4 translocation to the plasma membrane is limiting glucose uptake in insulin resistant states (Garvey et al., 1998) and, whilst this was not measured in the present study, it seems likely that GLUT4 translocation would also be impaired following fasting.

The changes in GLUT4 expression occurred in the absence of any change in the mRNA and protein expression of calpain-10. A number of studies have focused on a possible link between calpain-10 and GLUT4 trafficking as a mechanism whereby calpain-10 may influence glucose uptake into insulin sensitive tissues as outlined in **chapter 1** (see 1.9.2.2). Specific inhibition of calpain-10 in adipocytes inhibited insulin stimulated GLUT4 translocation via reduced actin reorganisation (Paul et al., 2003). A reduction in calpain-10 with fasting may lead to insulin resistance via a similar reduction in GLUT4 translocation and

possibly an accumulation of GLUT4 protein. Importantly, however, such a specific interaction between GLUT4 and calpain-10 has not been demonstrated in skeletal muscle *in vitro* or *in vivo*, although some indirect evidence does exist to support this idea. Otani et al. (2004) demonstrated that overexpression of the highly specific calpain inhibitor calpastatin resulted in a 3-fold increase in GLUT4 protein in the skeletal muscle of transgenic mice and that GLUT4 was a calpain substrate *in vitro*. In contrast to the hypothesis, calpain-10 was unchanged with fasting and refeeding whilst GLUT4 expression was downregulated, suggesting that calpain-10 expression is not important for skeletal muscle GLUT4 expression in insulin resistant states. One limitation of the present study is that measurements of calpain-10 activity were not obtained and cannot be measured at present. This is an important point because it is possible that changes in the expression of the ubiquitous calpains may not be necessary for their activity. If this is true for calpain-10, then perhaps a change in expression may not be expected during fasting and, instead, a reduction in calpain-10 activity may be limiting GLUT4 translocation, which unfortunately was not measured in this study. This model may not be consistent with the idea that GLUT4 is a calpain-10 substrate or with the observed reduction in GLUT4 content however, as a reduction in calpain-10 activity would be expected to lead to an increase in GLUT4 protein. Clearly, more detailed *in vitro* work using specific inhibition of calpain-10 in skeletal muscle is needed to clarify the role of calpain-10 in GLUT4 mediated pathways.

The primers and probe set used to assess calpain-10 mRNA hybridised to a region common to many of the calpain-10 mRNA isoforms and therefore the data represent the summative expression of five calpain-10 isoforms (a-e). It is technically very difficult to measure the expression of individual calpain-10 isoforms and it has been shown in any case that calpain-10a represents the majority of the calpain-10 message in skeletal muscle (Yang et al, 2001). Consideration of the expression of calpain-10 isoforms is important because some studies have indicated that smaller calpain-10 isoforms may be more physiologically relevant (Logie et al., 2005, Marshall et al., 2005). In pancreatic β -cells for example, it has been suggested that a smaller 54 kDa calpain-10 isoform is a regulator of insulin exocytosis (Marshall et al., 2005) and in human primary muscle cell cultures a 60 kDa calpain-10 band is increased with muscle cell differentiation (Logie et al., 2005). It is important to note that the antibodies used in these studies have not been fully characterised in human tissues and are designed to cross react with rat and not human calpain-10 (Ma et al., 2001), as is the focus of this thesis. Moreover, the immunoreactive bands were not sequenced in these studies making it impossible to rule out non-specific binding of the antibody. All of the bands recognised by the N7 antibody in this chapter similarly have not been sequenced and non-specific binding also cannot be ruled out, although the tests performed in this chapter go some way to provide evidence that this antibody is specific to human calpain-10.

What the observed calpain-10 bands represent is currently unknown. In calpain-10 knock-out mice, it was clear that there was at least one other band at 25 kDa, in addition to the full length protein, that cross reacted with the antibody. In human skeletal muscle, there was a similar sized band at approximately 30 kDa. This band may represent calpain-10f as this isoforms has been shown to contribute to the total calpain-10 expressed in skeletal muscle (Yang et al., 2001). The 60 kDa band observed on Western blots in this chapter similarly may represent isoforms 10b, 10c or 10d as their estimated molecular weights are close to 60 kDa. Alternatively these bands may represent post-translational proteolytic cleavage and/or autolysis of the full length 75 kDa protein, a common feature that occurs throughout the calpain system. It is also plausible that these bands indicate activation of calpain-10 *in vivo* in a similar manner to that described for calpain-3 autolysis (Kinbara et al., 1998). Based on the present study however, alternative protein isoforms of calpain-10 do not appear to play a significant role in the adaptive response to fasting and refeeding as they were not affected by fasting and refeeding.

There are a number of potential explanations as to why skeletal muscle calpain-10 expression was not affected by fasting and refeeding in the present study. Firstly, it could be argued that the magnitude of the physiological insult at the level of the skeletal muscle was not sufficient to observe a change in calpain-10 expression in this tissue. This is possible given that the ITT only provides a rough estimate of overall

tissue responsiveness to insulin and does not allow for a quantitative measurement of insulin mediated glucose metabolism, although it has been used successfully to screen for the presence of insulin resistance. The relative contribution of peripheral (skeletal muscle) and hepatic tissues to the reduction in insulin sensitivity seen with fasting is therefore not known and, as a result, the magnitude of insulin resistance in skeletal muscle could have been overestimated in the present study. However evidence against this suggestion comes from a previous study which used an almost identical fasting protocol and demonstrated that during a hyperinsulinaemic-euglycaemic clamp that there was a marked reduction in glucose uptake by the forearm muscle, indicating skeletal muscle insulin resistance (Mansell & Macdonald, 1990). Furthermore, it is possible that fasting induced insulin resistance is primarily a result of changes in genes important for glucose and fat oxidation and, in the short-term, may not necessarily be a result of a downregulation of insulin signaling pathways. Indeed fasting has been associated with an increase in the transcription and/or mRNA content of several genes important for lipid metabolism, including lipoprotein lipase (LPL) and CPT1 (Ladu et al., 1991; Hildebrandt and Neuffer, 2000; Pilegaard et al., 2003a). The regulation of the pyruvate dehydrogenase complex (PDC) has also been studied in response to starvation in both rodents and humans. Fasting causes a significant increase in PDK4 mRNA and protein content in rats, a response which is reversed by refeeding (Sugden et al., 2000; Wu et al., 1999; Wu et al., 1998). In humans, similar responses have been seen (Pilegaard et al., 2003a;

Spriet et al., 2004). In contrast, initial steps in the insulin signaling pathway have previously been shown to be unchanged by fasting in rats (Saad et al., 1992). The phosphorylation of AKT/PKB is unchanged by fasting in the skeletal muscle of chicks (Nakashima et al., 2006) and a two-day very low energy diet in obese subjects was not associated with changes in the expression of the insulin receptor or IRS1, or on PI3K activity (Jazet et al., 2005). Given the proposed role of calpain-10 in insulin signaling pathways, under these circumstances, one may not expect to observe a change in calpain-10 expression with fasting and refeeding. A further explanation could be related to circumstances under which measurements of skeletal muscle calpain-10 mRNA and protein expression were made. Muscle biopsies were obtained under basal (fasted) conditions during the 48 h fasting protocol and under postprandial conditions following refeeding. It would have been interesting to determine calpain-10 expression under hyperinsulinaemic conditions following fasting and refeeding and it is possible that insulin stimulated calpain-10 mRNA and protein expression would have been different following fasting and refeeding. This point is also relevant when considering the interaction of calpain-10 and GLUT4 mentioned above; calpain-10 inhibition has only been demonstrated to reduce GLUT4 translocation under insulin stimulated conditions in adipocytes. It is possible that a reduction in calpain-10 expression may have been seen if insulin clamps were performed following fasting.

To date only a single study has described an association between insulin resistance and calpain-3 expression (Walder et al., 2001). In this study, skeletal muscle calpain-3 mRNA was negatively correlated with fat mass and fasting glucose in non-obese human subjects, and with circulating insulin and glucose levels in *Psammomys obesus*. There was no effect of fasting-induced insulin resistance and its reversal on calpain-3 mRNA or protein levels. This observation further highlights the difference between the presence of a nutrient-gene association, which might not necessarily imply a cause and effect relationship, and a functional nutrient-gene interaction under conditions of altered nutrient supply.

As with calpain-10, measurement of calpain-3 protein revealed multiple bands representing the full length protein and a number of autolysis products. Interestingly, Western blot analysis revealed no significant increase in the appearance of calpain-3 autolysis products throughout the intervention. Given that there is strong evidence supporting the notion that calpain-3 autolysis products are indeed markers of activity, significant accumulation of these products was expected if calpain-3 activation was evident. Kinbara et al. (1998) documented the appearance of autolysis products at 60, 58 and 55 kDa, which were shown to originate from a unique insertion sequence (IS1) region of calpain-3, which is thought to be responsible for calpain-3 instability (Kinbara et al., 1998). More recent analysis in skeletal muscle myoblasts has shown that this autolysis is critical for the activity of

calpain-3 (Taveau et al., 2003). Expression of wild-type active calpain-3 in myoblasts led to profound morphological changes as a result of a disorganisation of the actin cytoskeleton and of focal adhesions (Taveau et al., 2003). Removal of the IS1 encoding region (exon 6) completely prevented the degradation and disorganisation of the actin cytoskeleton and focal adhesions indicating that calpain-3 autolysis is required for calpain-3 function (Taveau et al., 2003). In porcine skeletal muscle *in situ*, it has previously been shown that the presence of full length calpain-3 is reduced 24 h postmortem (Parr et al., 1999). In human (Murphy et al., 2006) and toad (Verburg et al., 2005) skeletal muscle, addition of calcium leads to the accumulation of autolysis products. Collectively, these data suggest that calpain-3 autolysis is linked to its activation as is common with other members of the calpain family. In the present study, however, a lack of accumulation of calpain-3 autolysis products on Western blots indicates that calpain-3 was not significantly activated. This finding is consistent with recent evidence from exercise studies supporting the idea that calpain-3 may not be important for glucose uptake into skeletal muscle (Murphy et al., 2006).

The failure to observe a change in calpain-3 in response to fasting may be related to its proposed role in proteolysis. It is not known in the current study whether significant proteolysis was observed in skeletal muscle and an absence of proteolysis may partially explain the lack of effect of fasting on calpain-3 mRNA and protein expression. Fasting

chicks (Nakashima et al., 2005) and lambs (Ilian et al., 2001) did lead to marked increases in skeletal muscle calpain-3 mRNA. In each of these studies, quantification of myofibrillar degradation revealed significant skeletal muscle proteolysis and this may explain the increased calpain-3 expression.

3.5 Conclusions

In the present study, fasting was associated with a reduction in whole body insulin sensitivity, as estimated using an ITT, and this was most likely associated with some degree of skeletal muscle insulin resistance; refeeding with a high carbohydrate diet restored whole body insulin sensitivity. It was hypothesised that fasting would lead to a reduction in calpain-10 mRNA and protein expression as reduced calpain-10 mRNA has previously been associated with insulin resistance in humans. However, fasting did not affect calpain-10 mRNA or protein levels, which were characterised in this study for the first time using a novel antibody. The mRNA and protein (in two subjects only) of the proposed calpain-10 substrate, GLUT4 was reduced with fasting despite no change in calpain-10 protein. More detailed studies are needed to investigate the effect of more pronounced skeletal muscle insulin resistance on calpain-10 expression. Furthermore, fasting may not be the ideal intervention to investigate the potential role of calpain-10 in insulin signaling pathways and studies that significantly affect the components of this pathway need to be performed. It is also not known

from the present study whether a change in the calpain-10 activity was responsible for the fall in GLUT4 and this is an interesting area for investigation. Calpain-3 mRNA and protein expression was similarly unchanged by fasting and refeeding and it is suggested that previous reports of an upregulation of calpain-3 with fasting may be related to changes in skeletal muscle proteolysis in those studies.

4 The effect of a one week high fat diet on insulin resistance and skeletal muscle calpain expression

4.1 Introduction

In **chapter 3** it was shown that short term fasting was associated with a reduction in whole body insulin sensitivity, alterations in serum insulin and blood glucose concentrations and a large increase in plasma FFA. These metabolic alterations were not associated with changes in the mRNA or protein expression of calpain-10 or -3 in skeletal muscle. However, as an insulin tolerance test (ITT) was employed in the previous chapter, it is not known whether the reduction in whole body insulin sensitivity seen was significant at the level of skeletal muscle, or was primarily an indication of hepatic insulin resistance.

Several studies have shown that when FFA availability was profoundly increased by Intralipid infusions, impaired whole-body insulin sensitivity was observed in humans. However, more physiological high-fat feeding in humans has produced contradictory results. Following 3 d of high fat feeding (73% fat content) in aerobically active human subjects, Pehleman and colleagues (Pehleman et al., 2005) noted a moderate but significant reduction in glucose disposal, as estimated using an OGTT. These findings were associated with an increase in basal (pre-OGTT) PDK activity and decreased PDH activation. During the OGTT,

GS activity and PDH activity increased to the same extent from baseline but PDH activity remained lower following the high-fat diet (Pehleman et al., 2005). Using the hyperinsulinaemic-euglycaemic clamp technique, 3 d of high-fat feeding (55-60% fat content) was also associated with a very moderate but significant reduction in insulin sensitivity (Bachmann et al., 2001). High-fat feeding for 11–21 d does not induce whole-body insulin resistance in healthy (Bisschop et al., 2001) and exercise-trained subjects (Cutler et al., 1995), although the partitioning of glucose metabolism is altered with decreased oxidation and increased nonoxidative glucose disposal. Therefore, more detailed *in vivo* studies are needed to investigate the potential changes in insulin mediated muscle metabolism and the underlying mechanisms that are associated with high dietary fat intake in humans.

It has been suggested that calpain-10 may be one gene that contributes to diabetes risk under certain environmental conditions, such as a switch to a diet high in fat. Pima Indians with the at-risk calpain-10 genotype at SNP-43 (G/G) have lower levels of total calpain-10 mRNA (Baier et al., 2000). Subjects with the at-risk allele at SNP-43 oxidised more lipid over a 24 h period (using a respiratory chamber) than those with the G/A and A/A genotype but the changes in the carbohydrate oxidation were not significant in this study. During the respiratory chamber studies, the subjects were fed calories to maintain energy balance during the 24 h period and therefore these data represent the total 24 h energy expenditure. Of particular interest, therefore, was that

basal (fasting) rates of carbohydrate and fat oxidation were no different between the two groups. During a hyperinsulinaemic-euglycaemic clamp, those with the at risk allele did oxidise significantly less carbohydrate than those without this genotype and had lower rates of glucose disposal as a result. Therefore, when provided with exogenous substrate, including lipid, carbohydrate and protein, subjects with the at risk allele (and lower calpain-10 mRNA expression) preferentially oxidise lipid. It was suggested by the authors that historically this reflects altered nutrient partitioning and allowed for an easier switch to ketone production in times of limited carbohydrate supply. It is also proposed that today (ample food supply) this phenotype is disadvantageous and “excludes” glucose oxidation and thus may contribute to insulin resistance and type 2 diabetes. On the contrary, recent preliminary data from transgenic mice lacking calpain-10 suggest that these mice are protected from a high fat diet mediated insulin resistance (Ye et al., 2006). These findings appear to contradict those of Baier et al. (2000), where a lower calpain-10 is associated with insulin resistance. However, on closer inspection, lower calpain-10 is associated with an increase in 24 h fat oxidation in that study and, from the discussions in chapter 1 (see 1.4), it is becoming clear that an impairment in fat utilisation is a major prerequisite for the development of insulin resistance. It is possible, therefore, that a reduced calpain-10 expression is beneficial during high fat feeding, although this has not been tested in humans. The mechanisms whereby calpain-10 may affect fat mediated insulin resistance are currently unknown but it is

possible that cleavage and activation of the calpain substrate PKC (1.3.1.4 and 1.8.2.5) and GLUT4 translocation (1.3.1.3) may be involved.

The predominantly muscle specific calpain-3 has also been linked to measures of insulin resistance in human and animal models. Walder et al. (2002) showed that in humans calpain-3 gene expression was negatively correlated with total and central abdominal fat mass and with blood glucose concentrations in non-obese subjects. In *Psammomys obesus*, an animal model of obesity, calpain 3 gene expression was negatively correlated with body fat mass and indirect calorimetry revealed associations between calpain 3 gene expression and carbohydrate oxidation and energy expenditure.

This study investigated the effect of 6 d isoenergetic high-fat/low carbohydrate diet (HF/LC) on insulin mediated whole body and muscle metabolism. In this study, a glucose tracer was used to accurately determine glucose turnover (including hepatic glucose output (Ra), glucose disposal (Rd) and non-oxidative disposal (NOD) during basal and insulin mediated states. To develop an understanding of nutrient gene interactions further, the impact of a HF diet on calpain-10 and -3 mRNA and protein expression was also investigated as these genes have been linked to insulin resistance associated with impaired fat metabolism. Based on the findings of Baier et al. (2000), it was hypothesised that the HF diet would induce insulin resistance and that

this would be associated with reduced skeletal muscle expression of calpain-10. Similarly, as calpain-3 mRNA has been negatively correlated with FFA, it was hypothesised that the HF/LC diet would reduce calpain-3 expression.

4.2 Subjects and Methods

4.2.1 Study protocol

Ten healthy male volunteers (age 25 ± 3 yr, body mass 78 ± 3 kg, BMI 24 ± 1 kg.m²) were recruited from amongst university students. Subjects were informed of all procedures and risks associated with the experimental procedures prior to obtaining informed consent. The subjects were medically screened as described in the **general methods (2.2.1)**, and were in good health prior to commencement of the study.

The study design is summarised in Fig 4.1. Subjects reported to the laboratory at approximately 8 am after an overnight fast and after recording the subjects' body weight, a baseline blood sample was taken for measurement of fasting glucose, serum insulin, plasma free fatty acids and ketone bodies (**general methods, 2.2.2**). Under local anaesthetic, the vastus lateralis muscle was then biopsied as described (2.2.7) (pre diet biopsy). Subjects were then randomised to either control (CON) or high fat (HF) diet described below for 6 days. Following the diet, on day 7, subjects reported to the laboratory again at 8 am after an overnight fast. Subjects were again weighed and a baseline urine sample was collected, before the subjects were allowed to rest on a bed. Resting $\dot{V}CO_2$ and $\dot{V}O_2$ was measured using a

metabolic cart for approximately 30 min as described (**general methods, 2.2.4**), before a second muscle biopsy sample was taken (post diet biopsy).

At approximately 9 am, a Teflon catheter was inserted into the antecubital vein of one arm for glucose tracer, 20% glucose (spiked with glucose tracer, 1%) and insulin infusions. Another catheter was placed into a dorsal hand vein in a retrograde fashion and placed in heated chamber at 55°C to obtain arterialized blood samples. Subjects were infused with [6, 6-²H₂]-glucose in a primed (4 mg/kg body mass)-continuous (2.5 mg/kg) fashion for 2 h before (basal period) and during a 4 h hyperinsulinaemic (serum insulin ~70 mU/l) euglycaemic (4.5mmol/l) clamp for the determination of glucose disposal (Rd) and glucose appearance (Ra). A second $\dot{V}CO_2$ and $\dot{V}O_2$ measurement was made during the last 30 min of the insulin glucose clamp (steady state) and a second muscle biopsy was obtained at the end of the insulin clamp (post insulin biopsy). Seven to eight millilitre blood samples were collected at 20 min intervals during the study for determination of serum insulin, plasma free fatty acids and ketone bodies and 5 ml arterialised venous blood samples were obtained at 105, 110, 115 and 120 min into the [6,6-²H₂]-glucose infusion and every 5 min during the last 20 min of the 4 h clamp for the determination of isotope enrichment (4.2.6). Urine samples were collected during the entire study day for the determination of glucose and nitrogen excretion.

4.2.2 Dietary intervention

Each subject consumed a HF and a CON diet for 6 days on two separate occasions with a minimum two-week interval between each diet. The HF and CON diets were designed based on the subjects' usual daily dietary habits and energy intake in order to increase the palatability of the diets and to maximise adherence to the study protocol. The HF diet was designed to provide 75% of the total energy from fat (35% of energy from saturated fat) and 10% of energy as carbohydrate. The CON diet on the other hand was designed to provide 50% of energy from carbohydrate and 35% of energy from fat. Each diet and its associated daily menu for the 6 day period was designed using a dietary analysis program (Microdiet, Version 1.1, April 2000, Downlee Systems Limited) and all food, beverages (non-caffeinated) and snacks for each diet were purchased and delivered to the door of the study participants. Written instructions on cooking methods and ingredients were also provided if the subject required them and ready-made meals with known nutritional content were provided to subjects who were unfamiliar with the cooking methods required. Subjects were asked to adhere strictly to the items on the menu and to record intake thoroughly, and they were also requested to abstain from alcohol consumption, smoking and intense exercise during both diet interventions. Post diet food records were analysed using the dietary analysis program.

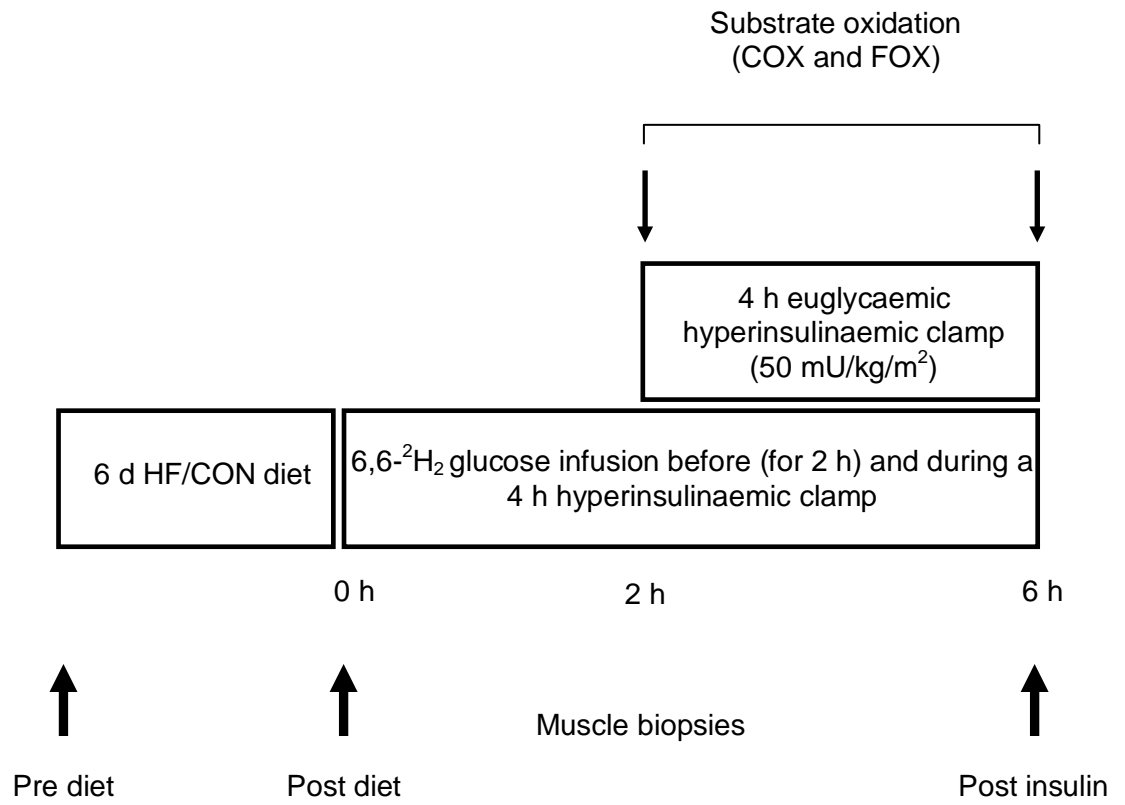


Figure 4.1 Schematic representation of the high fat study protocol.

Skeletal muscle biopsies were taken before each diet and before and after the insulin clamps following each diet. Rates of CHO oxidation (COX) and fat oxidation (FOX) were determined for 30 min before and at the end of the clamp. Blood samples were collected at 20 min intervals for determination of blood metabolites and at 105, 110, 115 and 120 min into the [6,6-²H₂]-glucose infusion and during the last 30 min of the 4 h clamp for the determination of isotope enrichment.

4.2.3 Blood and urine analysis

Collected blood samples were analysed for blood glucose, lactate and ketone bodies and serum insulin and plasma FFA as described under the **general methods (2.2.2)**. Urine was also analysed for nitrogen, also as previously described (**general methods, 2.2.3**). Plasma [6,6-²H₂]-glucose enrichment was determined by electron ionization gas chromatography-mass spectrometry analysis of the penta-acetate derivatives on a Finnigan INCOS-XL (Bremen, Germany). Glucose concentrations in the infusates were determined with the COBAS FARA semi-automatic analyzer. This work was performed by Dr Luc Van Loon at Maastricht University, The Netherlands).

4.2.4 Skeletal muscle biopsy analysis

Skeletal muscle biopsies were analysed for calpain-10 and -3 mRNA and protein expression using RT-PCR and western blotting procedures, as described in the **general materials (2.2.7.4)**.

4.2.5 Substrate oxidation rates

Rates of carbohydrate (COX) and fat oxidation (FOX) were obtained prior to the insulin clamps from the $\dot{V}\text{CO}_2$ and $\dot{V}\text{O}_2$ measurements as described in the **general methods (2.2.4)**. Insulin mediated oxidation

rates were calculated at steady state during the insulin clamp (last 30 min) in the same way.

4.2.6 Glucose disposal calculations

Calculations of glucose disposal from the glucose tracer data were performed at steady state during the last 30 min of the insulin clamp. Stable isotopes are non-radioactive atoms of the same chemical element which differ only in their number of neutrons. Following administration, the tracer is metabolically indistinguishable from the equivalent unlabelled compound of interest (tracee). The metabolic fate of the compound can be assessed quantitatively by measuring the relative abundance of tracer and tracee with time. The detectable mass difference of tracer and tracee allows for analysis of compounds, extracted from plasma, by gas chromatography-mass spectrometry. The glucose stable isotope dilution technique was used to determine glucose turnover during basal and insulin stimulated states.

Modified Steele equations were used to calculate glucose disposal (R_d) and glucose appearance (R_a) as described by Finegood et al. (1987). Hepatic glucose output was calculated as the difference between R_a and glucose infusion rate (GIR) during the clamp. Non-oxidative glucose disposal was calculated as the difference between R_d and COX.

4.2.7 Statistics

Data were analysed as described in the **general methods (2.6)**.

4.3 Results

4.3.1 High fat dietary analysis

The subjects maintained detailed food records and analysis of food intake revealed a small but significant excess energy intake in the HF group when compared to CON ($P < 0.01$, Table 4.1), although this did not lead to changes in body mass or resting energy expenditure. The mean daily proportion of energy as CHO was $7.4 \pm 0.2\%$ in the HF diet and $49.8 \pm 0.8\%$ in the CON diet ($P < 0.01$), whereas the fat intake was 76.7 ± 0.4 vs. $32.3 \pm 0.7\%$ ($P < 0.01$), respectively. There was no difference in protein intake between the two diets. All subjects experienced symptoms of lethargy and hunger during the HF dietary treatment.

4.3.2 Blood metabolites and hormones

Fasting blood glucose and insulin concentrations before (pre) and after (post) each diet were not significantly different (Table 4.2). Whilst plasma FFA concentrations were similar before and after the CON diet, high fat feeding increased fasting FFA concentrations, although this increase did not reach significance (Table 4.2). Fasting β -Hydroxybutyrate concentrations were unchanged by the CON diet but were increased following the HF diet ($P = 0.05$) (Table 4.2). Insulin

(CON 71.8 ± 3.5 vs. HF 70.0 ± 3.5 mU/L, Fig 4.2C) and glucose concentrations (CON 4.43 ± 0.05 vs. HF 4.44 ± 0.04 mmol/L, Fig 4.2A) were similar, and plasma FFA concentrations (CON 0.01 ± 0.003 vs. HF 0.01 ± 0.006 mmol/L, Fig 4.2B) were suppressed to a similar extent in both trials at steady state during the hyperinsulinaemic clamp (Fig 4.2).

Table 4.1 Energy and nutrient intake following CON and HF diets (n = 10).

	CON	HF
Energy Intake (Kcal/kg/day)	34.4 ± 1.5	39.9 ± 1.9**
Carbohydrate (g)	351 ± 12	62 (3)
% of total	49.8 ± 0.8	7.4 ± 0.2**
Protein (g)	116 ± 3	121 ± 4
% of total	17.9 ± 0.6	15.9 ± 0.4
Fat (g)	95 ± 3	259 ± 6
% of total	32.3 ± 0.5	76.7 ± 0.4**

**P<0.01 compared to control values.

Table 4.2 Blood metabolite concentrations before (pre) and after (post) the CON and HF diets (n = 10).

	CON		HF	
	Pre	Post	Pre	Post
Blood Glucose (mmol/l)	4.8 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.4 ± 0.2
Serum Insulin (mU/l)	7.4 ± 1.3	6.4 ± 0.8	6.1 ± 0.6	5.1 ± 0.7
Plasma FFA (mmol/l)	0.47 ± 0.08	0.48 ± 0.04	0.42 ± 0.04	0.53 ± 0.04
Blood β- hydroxybutyrate (mmol/l)	0.08 ± 0.02	0.13 ± 0.03	0.07 ± 0.01	0.56 ± 0.19*

*P=0.05 vs. pre diet values.

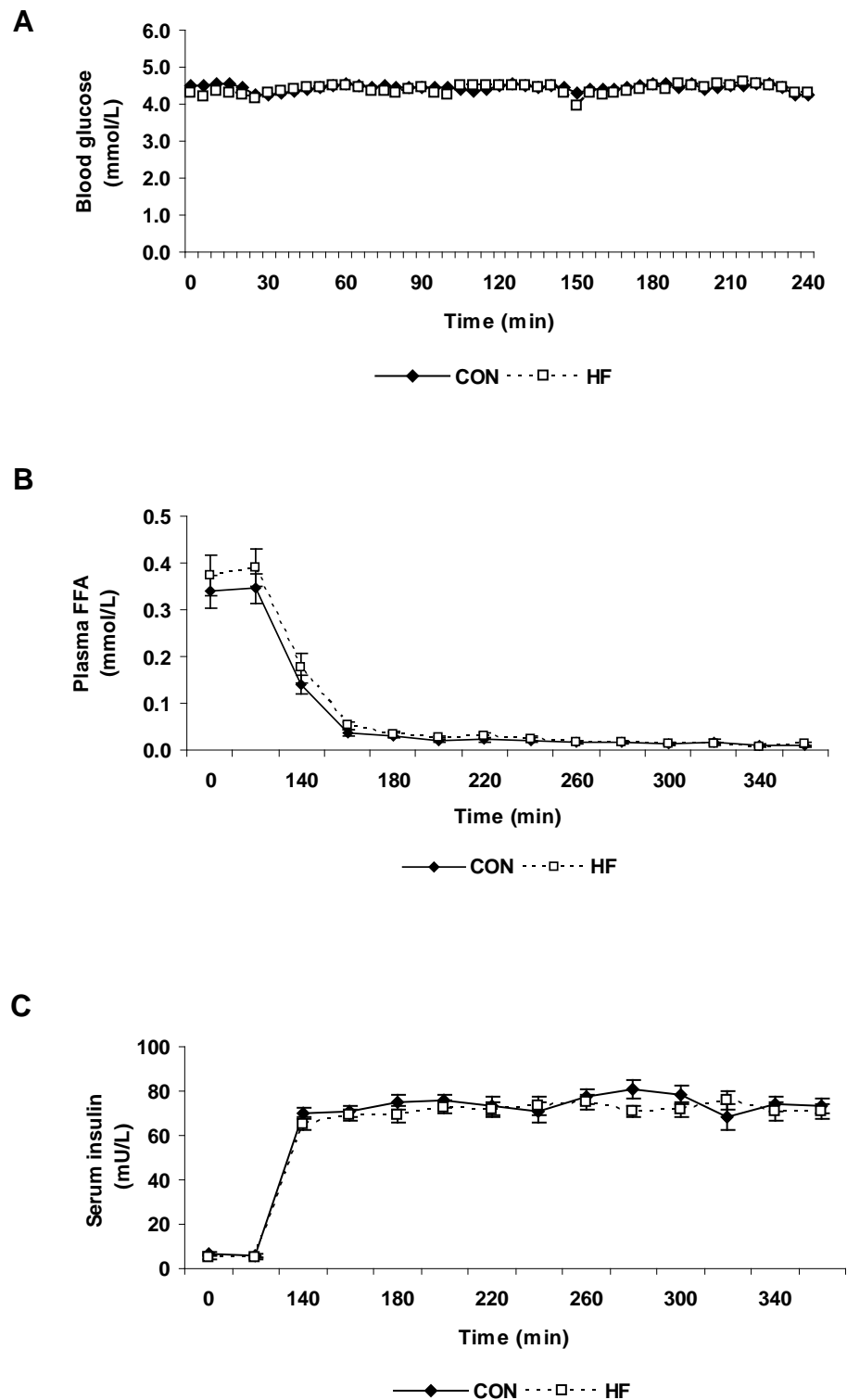


Figure 4.2 Blood glucose (A), plasma FFA (B) and serum insulin concentrations (C) during the insulin clamp before and after the CON and HF diets (n = 10).

4.3.3 Whole body substrate metabolism

There was no difference in glucose disposal (R_d) between diets under basal, non-insulin-stimulated conditions (CON 10.6 ± 2.6 vs. HF 8.8 ± 2.9 $\mu\text{mol/kg/min}$, Fig 4.3A). However, under insulin clamp conditions there was an increase in R_d during the last 30 min of the 4 h clamp (CON 57.5 ± 3.8 vs. HF 64.5 ± 4.9 $\mu\text{mol/kg/min}$, $P < 0.05$) (Fig 4.3B). On the other hand, COX was impaired after HF when compared to CON under basal (CON 8.0 ± 1.2 vs. HF 4.6 ± 1.4 , $P < 0.05$) and insulin stimulated conditions (CON 21.5 ± 2.4 vs. HF 17.2 ± 1.0 $\mu\text{mol/kg/min}$, $P < 0.05$) (Fig 4.3). High fat feeding increased non-oxidative glucose disposal (NOD) under basal (CON 2.98 ± 1.07 vs. HF 4.39 ± 1.19 $\mu\text{mol/kg/min}$) and insulin stimulated conditions (CON 36.0 ± 2.5 vs. HF 47.3 ± 4.6 $\mu\text{mol/kg/min}$, $P < 0.05$) (Fig 4.3). Hepatic glucose output was comparable under basal conditions following both diets (CON 10.9 ± 0.9 vs. HF 8.8 ± 0.8 $\mu\text{mol/kg/min}$) and was completely suppressed during insulin infusion in both trials (Fig 4.3).

Subjects were found to oxidise significantly more fat during the insulin clamp following the HF diet compared to CON (CON 3.3 ± 0.5 vs. HF 5.3 ± 0.3 $\mu\text{mol/kg/min}$, $P < 0.05$, Fig 4.3B). However, whilst there was a trend for increased fat oxidation under basal conditions following HF, this did not reach significance (CON 7.2 ± 0.2 vs. HF 8.7 ± 0.7 $\mu\text{mol/kg/min}$, $P = 0.06$) (Fig 4.3).

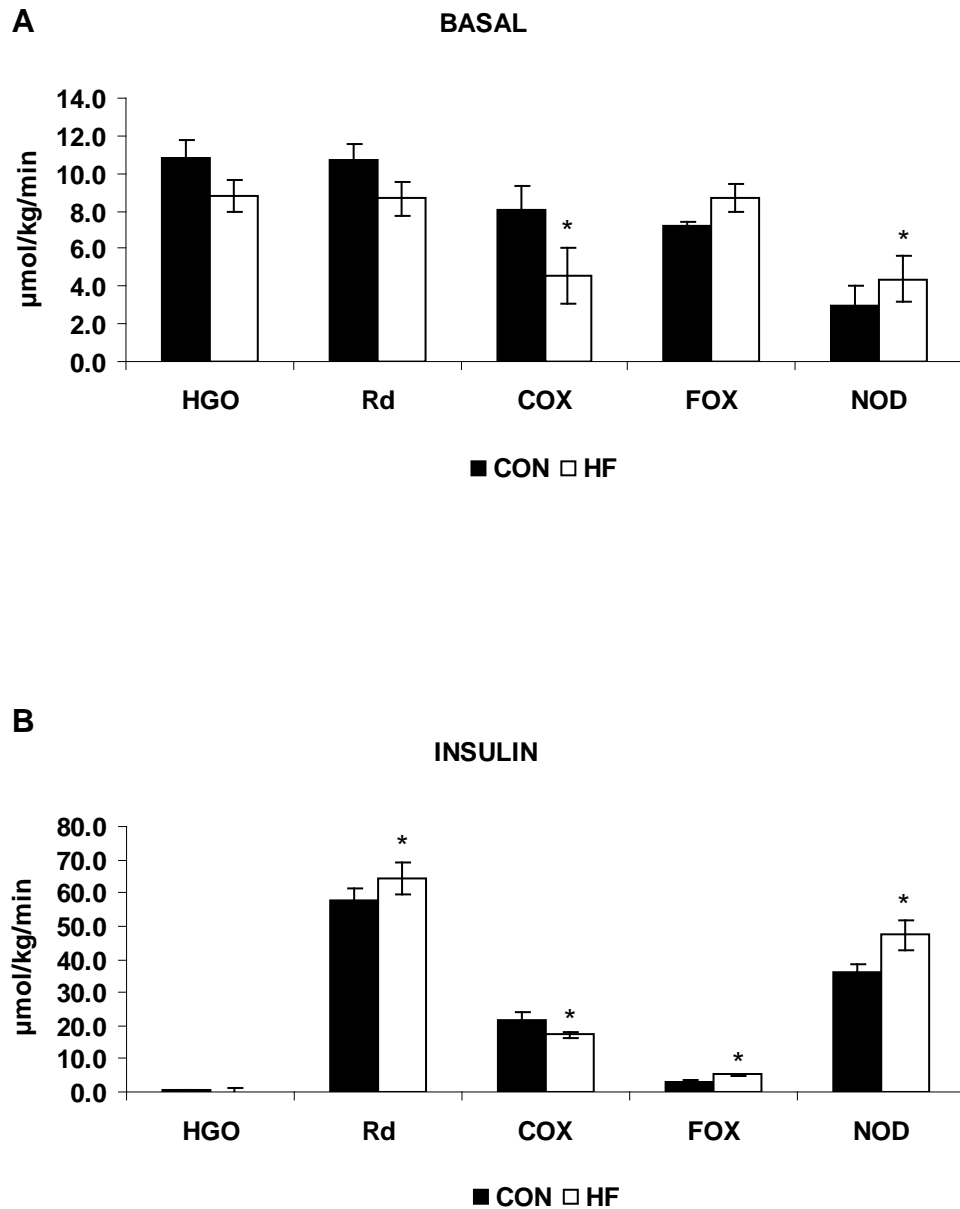


Figure 4.3 The effect of CON and HF diets on whole body glucose and fat metabolism ($\mu\text{mol/kg/min}$) under basal (pre clamp) (A) and insulin stimulated conditions (B) ($n = 10$).

Hepatic glucose output (HGO), glucose disposal (Rd), glucose oxidation (COX), fat oxidation (FOX) non-oxidative disposal (NOD) under basal and insulin stimulated conditions following the CON and HF diets. * $P < 0.05$ vs. CON value.

4.3.4 Calpain-10 expression

Analysis of calpain-10 mRNA revealed no effect of diet (PRE CON 1.2 ± 0.2 vs. POST CON 1.3 ± 0.4 and PRE HF 1.2 ± 0.2 vs. POST HF 1.2 ± 0.2) or insulin infusion following each diet (POST CON 1.3 ± 0.4 vs. POST INSULIN CON 1.1 ± 0.2 and POST HF 1.2 ± 0.2 vs. POST INSULIN HF 0.9 ± 0.1) (mixed model analysis: diet effect, $P=0.893$; time effect, $P=0.238$; diet*time interaction, $P = 0.880$) (Fig 4.5). A small statistical trend towards reduced full length 75 kDa calpain-10 protein was observed with insulin infusion (POST CON 0.75 ± 0.12 vs. POST INSULIN CON 0.40 ± 0.09 and POST HF 0.62 ± 0.13 vs. POST INSULIN HF 0.55 ± 0.12) (mixed model analysis: insulin effect, $P=0.110$) and this was independent of any diet effect (Fig 4.6). There was no effect of diet or insulin on the density of the 60 kDa immunoreactive band (Fig 4.6).

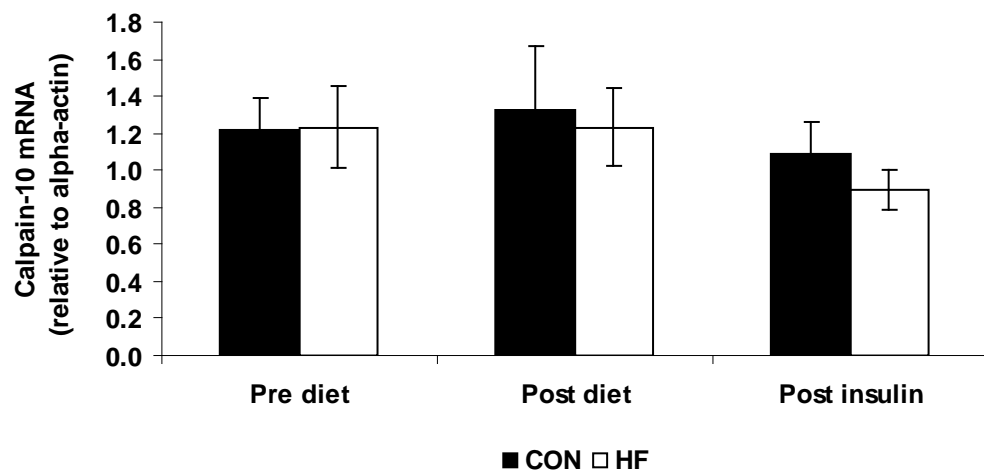


Figure 4.4 The effect of the CON and HF diets and insulin infusion on skeletal muscle calpain-10 mRNA expression (n = 10).

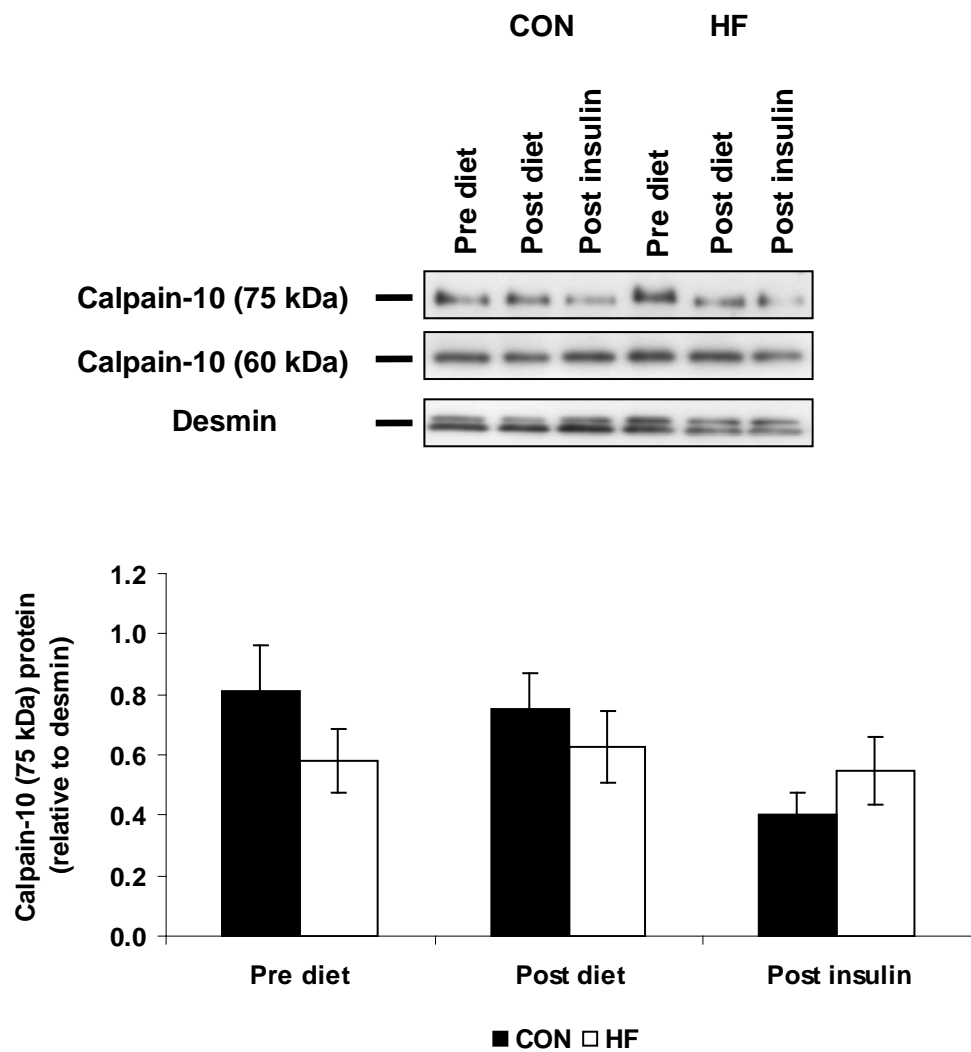


Figure 4.5 Effect of the CON (n = 10) and HF diets (n = 9) and insulin infusion on skeletal muscle calpain-10 (75 kDa) protein expression

A representative Western blot for calpain-10 (75 and 60 kDa) and the corresponding blot for the control protein (desmin, 55 kDa) is shown. Calpain-10 and desmin were determined on the same Western blot.

4.3.5 Calpain-3 expression

Calpain-3 mRNA was not affected by either diet (PRE CON 1.2 ± 0.3 vs. POST CON 1.0 ± 0.2 and PRE HF 0.9 ± 0.1 vs. POST HF 1.0 ± 0.2) or insulin infusion following each diet (POST CON 1.0 ± 0.2 vs. POST INSULIN CON 1.0 ± 0.2 and POST HF 1.0 ± 0.2 vs. POST INSULIN HF 0.9 ± 0.1) (Fig 4.8). Similarly, there was no effect of diet (PRE CON 1.8 ± 0.3 vs. POST CON 1.1 ± 0.2 and PRE HF 1.2 ± 0.1 vs. POST HF 1.0 ± 0.1) or insulin (POST CON 1.1 ± 0.2 vs. POST INSULIN CON 1.4 ± 0.4 and POST HF 1.0 ± 0.1 vs. POST INSULIN HF 1.7 ± 0.4) on calpain-3 protein expression (Fig 4.9). There was no significant accumulation of calpain-3 autolysis products as shown in Fig 4.9 and quantification of any visible autolysis products was not performed due to their very low abundance.

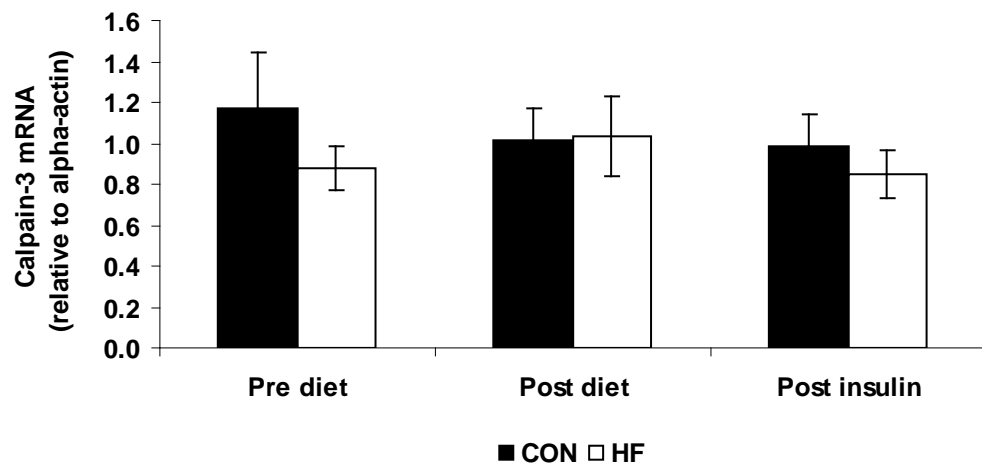


Figure 4.6 Effect of the CON and HF diets and insulin infusion on skeletal muscle calpain-3 mRNA expression (n = 10).

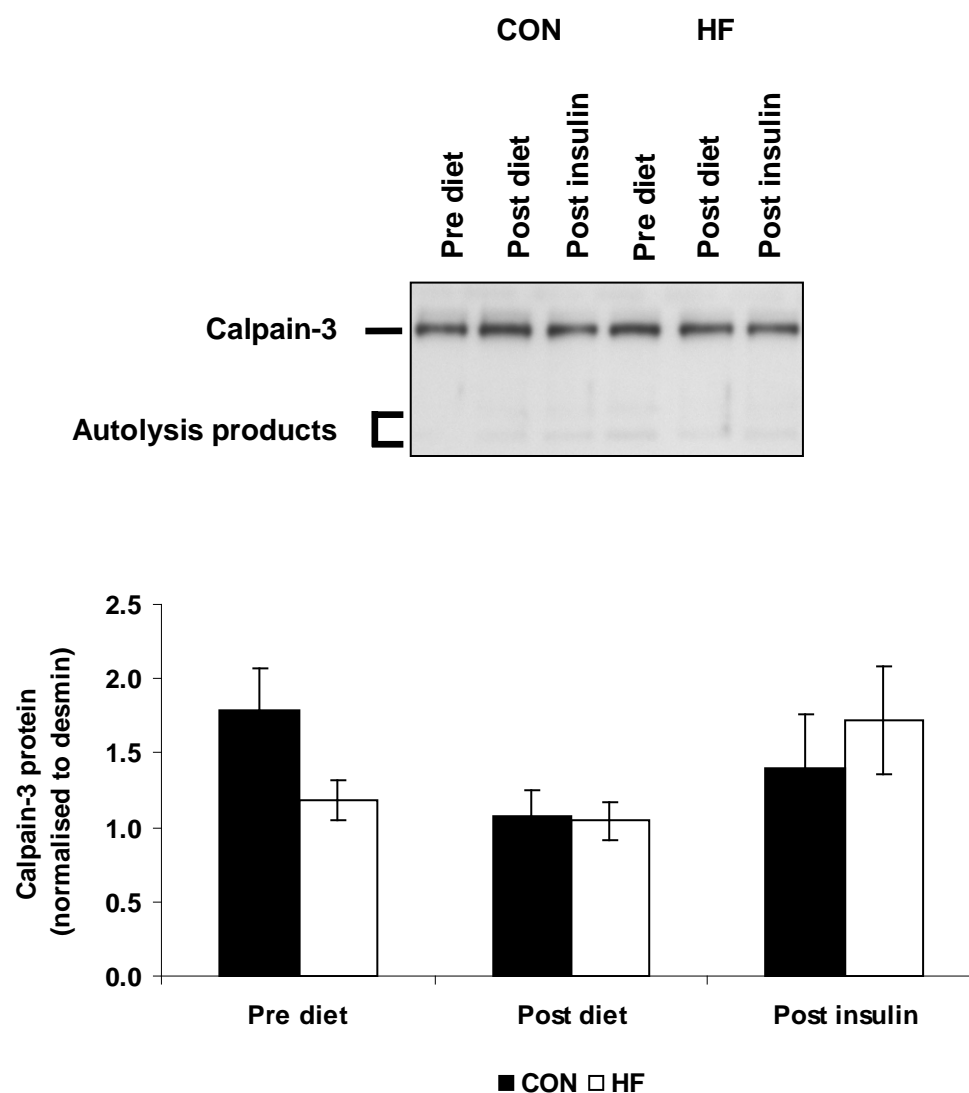


Figure 4.7 Effect of the CON and HF diets and insulin infusion on skeletal muscle calpain-3 protein expression (n = 10).

A representative blot for calpain-3 (94 kDa) and calpain-3 autolysis products (55 – 60 kDa) is shown. Autolysis products of calpain-3 were not quantified due to their very low abundance in the present study.

4.4 Discussion

The main findings in this chapter are that high fat feeding did not affect the expression of calpain-10 or -3 mRNA and protein expression. Whilst this is the first study to examine the effect of a 6 d high fat diet on calpain-10 and -3 expression in skeletal muscle, the diet employed in the current study did not have a significant negative impact on basal or insulin mediated glucose disposal. Instead, the high-fat diet tended to increase the insulin mediated Rd and led to only moderate reductions in basal and insulin stimulated COX and FOX. Crucially, these findings may explain the lack of effect of the HF diet on calpain expression.

High fat feeding studies in humans have produced contradictory results. Studies employing a HF diet for shorter periods of time have documented small reductions in glucose disposal when using the oral glucose tolerance test (OGTT) to estimate insulin sensitivity. For example, 3 d of a high-fat (73% fat content), low carbohydrate diet reduced whole body glucose disposal during an OGTT (Pehleman et al., 2005). A similar increase in the blood glucose-area under the curve (AUC) during an OGTT has been documented following a 5 d diet high in fat (53% fat content) (Anderson and Herman, 1975). Whilst these studies suggest that short-term high fat diets induce insulin resistance, the OGTT is an index of whole body glucose disposal and includes a component made up of hepatic glucose output and hepatic glucose disposal. During an OGTT, hepatic glucose disposal can account for

~25% of the glucose disposal and hepatic glucose output is only reduced by ~50% (Katz et al., 1983). As such, the results from these studies cannot be readily compared to the data obtained using the hyperglycaemic-euglycaemic clamp in the present study. One study that did employ a hyperinsulinaemic-euglycaemic clamp following a short-term high-fat diet did report a small but significant decrease in glucose disposal (~85% of control) (Bachmann et al., 2001).

On the other hand, high fat dietary treatment for 11 to 21 days does not induce whole body insulin resistance, although the partitioning of glucose metabolism is altered with decreased oxidation and increased non-oxidative glucose disposal (Bisschop et al 2001; Cutler et al., 1995). Furthermore, a study employing a high fat diet for 16 days reported no effect on glucose infusion rate (GIR) during a 3 h hyperinsulinaemic-euglycaemic clamp (Yost et al., 1998). This is consistent with data from the present study as a slight increase in Rd during the last 30 min of the clamp was observed after the HF diet when compared to the CON. It is possible that acute changes in dietary fat availability (several hours up to 3 days) might induce insulin resistance because of a greater imbalance between plasma FFA availability and their muscle oxidation, whereas after several days an increase in FFA availability can be compensated by a greater intramuscular lipid storage and/or utilisation. Alternatively, methodological differences in determining insulin resistance (i.e. OGTT vs. hyperinsulinaemic-euglycaemic clamp, clamp duration and insulin concentrations) could

explain the difference between these studies. Further studies are required to elucidate the precise sequence of adaptations to high-fat diets in humans.

These minor physiological effects of the high fat diet in the present study were not associated with changes in the expression of genes calpain-10 and -3 and these results are consistent with those in **chapter 3**. Previous studies have suggested a link between calpain expression and carbohydrate metabolism in human and rodent models (Baier et al., 2000; Walder et al., 2002; Carlsson et al., 2005). In Pima Indians, genetic variation in the calpain-10 gene at SNP-43 is linked to considerably lower levels (>50%) of skeletal muscle calpain-10 mRNA (Baier et al., 2000). The individuals with lower levels of calpain-10 were found to oxidise more lipid and less protein over a 24 h period when compared to those individuals with higher levels of calpain-10 mRNA. Moreover, a statistical trend towards reduced COX in those with lower levels of skeletal muscle calpain-10 was seen over the same 24 h period (Baier et al., 2000). Whilst the small reduction in COX seen in the present study was not associated with a reduction in calpain-10 mRNA and protein levels, it is possible that the physiological insult was not significant enough to drive a transcriptional/translational change in calpain-10. Thus it is currently difficult to rule out a role for calpain-10 in the physiological adaptation to a high-fat diet.

Whilst the exact metabolic adaptations to a high-fat diet are currently unknown, it is unlikely that the diet in the present study elicited a significant effect on insulin signalling and GLUT4 translocation, although this was not determined here. In support of this, 3 d of high-fat diet did not effect glycogen synthase (GS) activity under basal conditions or during an OGTT, suggesting that insulin signalling remained intact following the dietary intervention and is not an early adaptation to high-fat availability. On the contrary, as with the metabolic adaptation to starvation, a number of studies have demonstrated an effect of high fat feeding on regulatory enzymes controlling carbohydrate oxidation. In a study performed by Peters et al. (2001), six male subjects were fed a eucaloric, high-fat diet consisting of 73% fat for 3 d and PDK2 and PDK4 mRNA and protein expression and PDK activity was determined. High-fat feeding increased PDK activity in a linear fashion from 1 to 3 d post high-fat diet and this was accompanied by an increase in PDK4 mRNA and protein within 1 d of high fat feeding (Peters et al., 2001). Similarly, a 56 h high-fat diet led to a increase in PDK activity and a reduction in basal PDH (Pehleman et al., 2005); the activation of PDH during an OGTT was also blunted following the high-fat diet (Pehleman et al., 2005). Together these studies suggest that the early adaptation to a short-term high-fat diet is characterised by an impairment in glucose oxidation pathways possibly preceded by changes in the expression and activity of enzymes important in the regulation of substrate utilisation. These findings have implications for the present study as it was hypothesised

that high-fat feeding would lead to insulin resistance characterised by a reduction in insulin mediated glucose uptake and this was clearly not seen in the present study. Considering this, it is perhaps not surprising that no change in calpain-10 expression was observed in the current study.

In order to observe a significant effect on glucose uptake and insulin signalling pathways, based on the present data, it is likely that exposure to a high-fat diet would need to be increased considerably. In support of this, animal models of high fat feeding have shown that insulin receptor function is normal in rats after 8-weeks of high fat feeding (Hansen et al., 1998). Shorter periods of high fat feeding (4-5 weeks) were also shown to have little or no effect on insulin binding (Boyd et al., 1990) and insulin stimulated autophosphorylation or tyrosine kinase activity of purified receptors (Boyd et al., 1990). However, recent studies have investigated the effect of high-fat feeding on downstream insulin signalling molecules in rats. Four weeks of high-fat feeding downregulated insulin stimulated PI3K activity and AKT/PKB phosphorylation and impaired GLUT4 translocation (Tremblay et al., 2001). The same study showed that the reduction in insulin stimulated GLUT4 translocation was associated with a complete failure of insulin to activate aPKC (see 1.2.1.5) (Tremblay et al., 2001). These studies suggest that longer periods of exposure to high-fat diets in rodents are associated with impairments in distal steps in the insulin signalling cascade.

The present study shows that calpain-3 mRNA and protein expression is unaffected by high fat feeding and insulin infusion in the skeletal muscle of healthy humans. There was also no increase in the accumulation of calpain-3 autolysis products. These data are in contrast to the only report to document an association of calpain-3 mRNA with phenotypes related to insulin resistance in humans and rodents (Walder et al., 2002). As with calpain-10, it remains possible that calpain-3 may play a posttranslational role in signalling mechanisms that are important for glucose/FFA utilisation; however investigations into the role of calpain-3 in this respect have been almost impossible due to the unstable nature (half life of minutes) of the protein as soon as it is purified in the absence of its associated myofibrillar structures. Using cell culture, human biopsies and mouse models, it has been possible to show that calpain-3 may be involved in processes as diverse as apoptosis, muscle cell differentiation and remodelling and regulation of the cytoskeleton (Krameroval et al., 2006).

4.5 Conclusions

In the present study, 6 d of high-fat feeding had no effect on calpain-10 and -3 mRNA and protein expression in human skeletal muscle. It was hypothesised that high-fat feeding would lead to significant insulin resistance and that this would be associated with reduced expression of calpain-10 and -3 expression. However, the dietary intervention was characterised by early physiological adaptations manifesting in only

small changes in carbohydrate and fat oxidation and not by defects in insulin stimulated glucose disposal, as hypothesised. These findings currently make it difficult to rule out a role for calpain-10 and -3 in lipid induced insulin resistance in the present study and further human *in vivo* studies that produce a larger physiological insult are needed to clarify the role of these proteases in the adaptation to high lipid availability.

5 Lipid infusion, insulin resistance and skeletal muscle calpain expression in healthy humans

5.1 Introduction

In **chapter 4**, a one-week high fat diet led to a modest increase in plasma FFA but did not lead to significant reductions in insulin sensitivity. More acute elevation of FFA availability, on the other hand, results in severe insulin resistance in healthy humans (Ferrannini et al., 1983; Boden et al., 1991). Acute elevations of plasma FFA are usually achieved via the simultaneous infusion of a triglyceride emulsion and heparin, which activates lipoprotein lipase activity and promotes intravascular lipolysis of the triglyceride emulsion. The effect of FFA on glucose disposal occurs well within the physiological range of FFA concentrations seen in, for example, obesity and has been shown to be dose dependent (Belfort et al., 2005). In contrast to short-term fasting and high-fat diets, acute elevation of FFA concentrations leads to a downregulation of the insulin signalling pathway and is associated with reduced insulin receptor and IRS-1 tyrosine phosphorylation, reduced PI3K activity associated with IRS-1 and impaired AKT/PKB serine phosphorylation (Belfort et al., 2005).

Carlsson et al. (2005) were the first to document the effect of intralipid infusion on skeletal muscle calpain-10 mRNA levels. In this study,

seven male subjects with impaired glucose tolerance (IGT) and family history of type 2 diabetics and eight control subjects with normal glucose tolerance (NGT) were infused with Intralipid for 0, 2 and 24 h on three separate occasions. These infusions were followed by a standard ($40 \text{ mU/m}^2/\text{min}$) 2 h hyperinsulinaemic-euglycaemic clamp in the continued presence of the Intralipid infusion. The authors found that calpain-10 mRNA was no different between the two groups prior to the insulin clamp. However, their main finding was that calpain-10 mRNA was increased with insulin infusion for 2 h following 24 h of Intralipid infusion in the NGT group when compared to the IGT group. Intralipid infusion alone had no effect on calpain-10 mRNA levels. Moreover, infusion of Intralipid for shorter periods of time (0 and 2 h) did not have the same effect and was no different before or after the insulin clamp in both groups.

This chapter extends this previously published work and the work in **chapter 4** of this thesis by investigating the effects of a 6 h infusion of a triglyceride emulsion (Intralipid) on insulin sensitivity and calpain-10 mRNA and protein levels. A change in calpain-10 expression with insulin resistance in the present study may be linked to changes in the GLUT4 expression and/or translocation, as both have been shown to be downregulated by elevated lipid availability (Vettor et al., 2002; Zierath et al., 1997). Therefore, the expression of skeletal muscle calpain-10 mRNA and protein and GLUT4 mRNA was determined in the present study and It was hypothesised that Intralipid infusion would lead to

insulin resistance and that this would be associated with a reduction in the expression of calpain-10 mRNA and protein and a reduction in GLUT4 mRNA content.

5.2 Subjects and Methods

5.2.1 Study protocol

Ten healthy men (age 22 ± 1 yr, body mass 78 ± 3 kg, BMI 24 ± 1 kg.m²) were recruited from the students of the University of Nottingham to take part in this study.

The study protocol is summarised in Fig 5.1. On two separate occasions, interspersed by a minimum of two weeks, subjects underwent hyperinsulinaemic-euglycaemic clamps for 6 h with or without the intravenous infusion of a 20% Intralipid emulsion (a commercial emulsion of soya bean oil) at 90 ml/h and heparin (200 U prime and 600 U/h). Subjects reported to the laboratory at approximately 8 am and after baseline measurements were made, a baseline muscle biopsy was taken, as described previously (**general methods, 2.2.7**). At approximately 9 am, a Teflon catheter was inserted into the antecubital vein of one arm for the infusion of 20% glucose, insulin and 20% intralipid plus heparin. Another catheter was placed into a dorsal hand vein in a retrograde fashion and placed in heated chamber at 55°C to obtain arterialized blood samples as before. Infusions were continued for 6 h and measurements of FOX and COX were taken every 45 minutes. Additional skeletal muscle biopsies were obtained half way through (3 h clamp biopsy) and at the end of the intravenous infusions (6 h clamp biopsy). As before for the high fat

study, blood samples were collected every 20 min for determination of serum insulin, plasma FFA and ketone bodies.

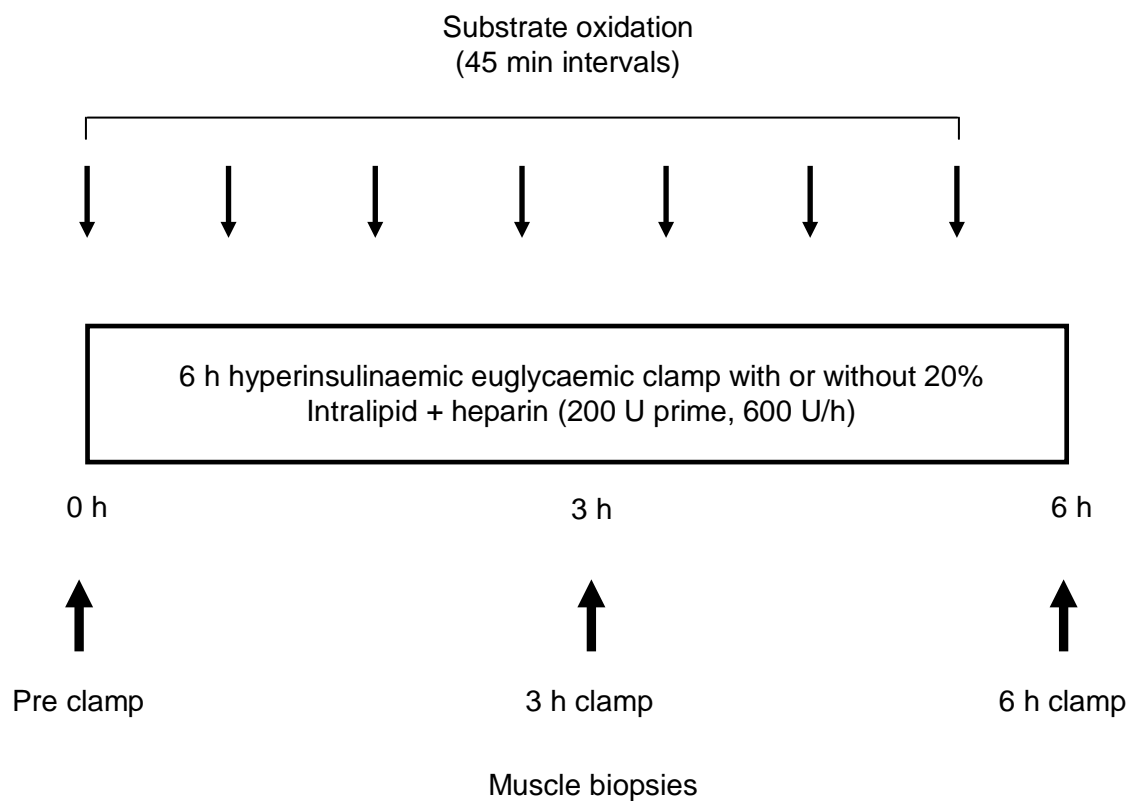


Figure 5.1 Schematic representation of the intralipid study protocol.

Skeletal muscle biopsies were taken before, during and at the end of the insulin clamp. Substrate oxidation rates were determined every 45 minutes during the clamp using indirect calorimetry. Blood samples were taken every 20 min for determination of blood metabolites.

5.2.2 Blood and urine analysis

Collected blood samples were analysed for blood glucose, serum insulin, plasma FFA and ketones as described in the **general methods (2.2.2)**. Urine was also analysed for urea, also as previously described (general methods).

5.2.3 Dual energy x-ray absorptiometry (DEXA)

Measurements of lean body mass and fat mass were determined for the whole body by DEXA scanning using an extended analysis program for body composition (Hologic QDR-2000, Waltham, MA, USA). The procedure was performed each time by the same trained technician (Elizabeth Simpson, School of Biomedical Sciences, University of Nottingham) who positioned the subjects, performed the scans, and completed the scan analysis according to the operator's manual using the standard analysis protocol. Quality-assurance tests were performed each morning of assessment.

5.2.4 Skeletal muscle biopsy analysis

Skeletal muscle biopsies were analysed for calpain-10 mRNA and protein expression and GLUT4 mRNA using RT-PCR and western blotting procedures, as described in the **general methods (2.2.7)**.

Skeletal muscle protein was extracted using the new extraction buffer B, as discussed in the **general methods (2.2.7)**. All mRNA results were normalised to actin expression as this is expressed in the supernatant fraction when using buffer B (see 2.2.7.6).

5.2.5 Substrate oxidation rates

Rates of COX and FOX were obtained prior to insulin clamps from the $\dot{V}CO_2$ and $\dot{V}O_2$ measurements as described (**general methods, 2.2.4**). During both insulin clamps, rates of COX and FOX were determined in the same way every 45 min as described in the **general methods (2.2.4)** chapter.

5.2.6 Glucose disposal calculations

Glucose disposal was calculated from the glucose infusion rate and normalised to the total lean body mass of each subject, as described previously in **chapter 4 (4.2.6)**.

5.2.7 Statistics

Data were analysed as described in the **general methods (2.6)**.

5.3 Results

5.3.1 Blood metabolites

At baseline, prior to infusions, plasma FFA (CON 0.46 ± 0.07 vs. LIPID 0.63 ± 0.11 mmol/L, Fig 5.2B), serum insulin (CON 8.7 ± 0.2 vs. LIPID 8.3 ± 0.2 mU/L, Fig 5.2C) and blood glucose (CON 4.6 ± 0.1 vs. LIPID 4.6 ± 0.1 mmol/L, 5.2A) were not different between the CON and LIPID trials.

FFA were elevated throughout the clamp in the LIPID trial (2.3 ± 0.3 mmol/L) but were completely suppressed in the CON trial (0.02 ± 0.003 mmol/L) (Fig 5.2B). The average blood glucose concentration during the clamp was similar in the two trials (CON 4.5 ± 0.02 vs. LIPID 4.6 ± 0.02 mmol/L, Fig 5.2A) and in each case was similar to the fasting glucose concentration. The mean serum insulin concentration during the clamp was not different between trials (CON 89.5 ± 2.7 vs. LIPID 96.5 ± 1.8 mU/L, Fig 5.2C).

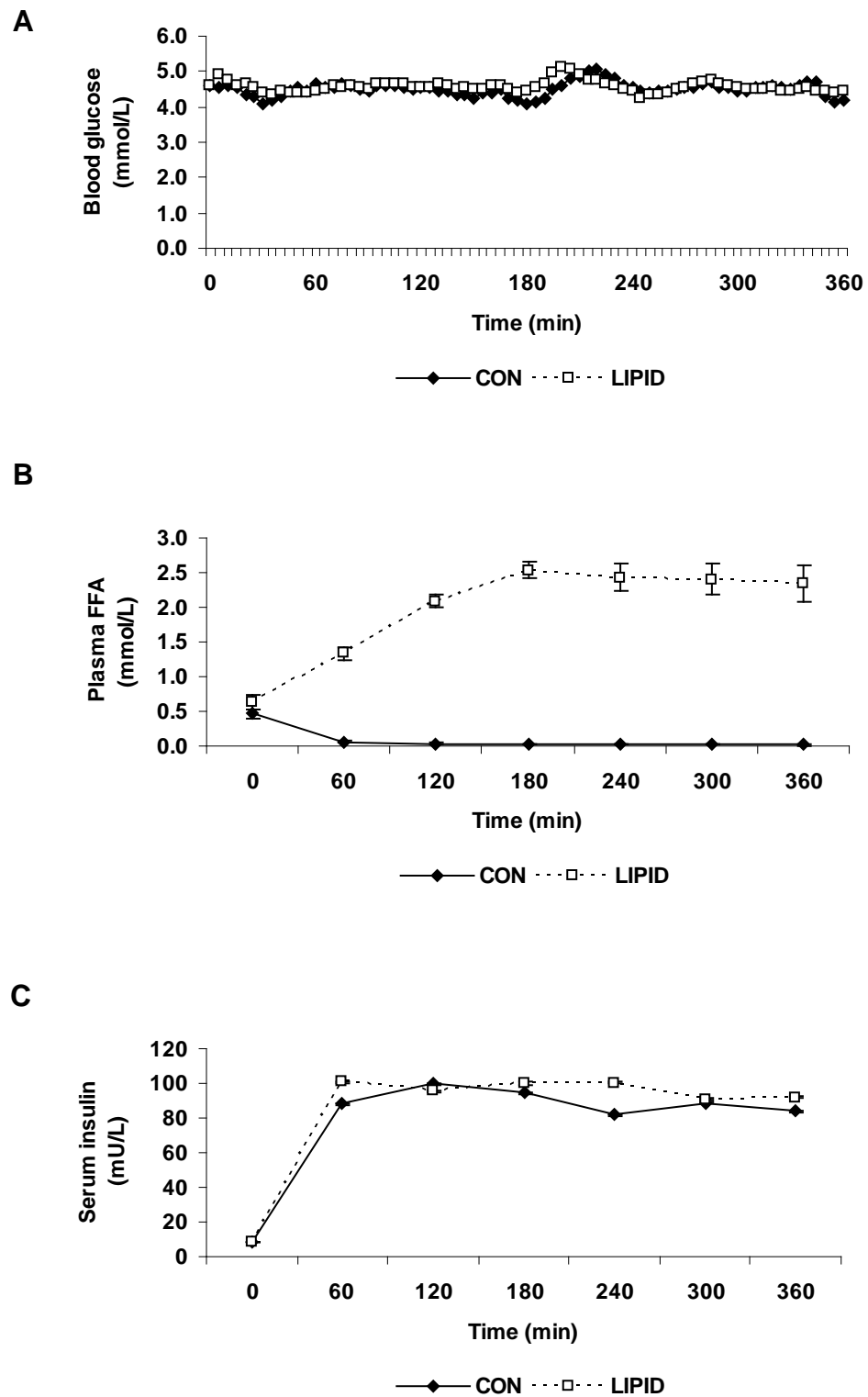


Figure 5.2 Blood glucose (A), plasma FFA (B) ($n = 10$) and serum insulin concentrations (C) ($n = 9$) during the insulin clamp with (LIPID) or without (CON) the simultaneous infusion of Intralipid.

5.3.2 Whole body substrate metabolism

Glucose disposal as calculated from the glucose infusion rate was reduced by approximately 50% in the LIPID trial (Fig 5.3A). During the last 30 min of the clamp, at steady state, glucose disposal was 79.6 ± 4.0 $\mu\text{mol/kg lean mass/min}$ in the CON trial compared to 37.8 ± 4.4 $\mu\text{mol/kg lean mass/min}$ in the LIPID trial ($P < 0.001$). Post-hoc analysis revealed that the reduction in glucose disposal began 210 min into the insulin clamp ($P < 0.05$) (Fig 5.3A).

The effect of Intralipid on glucose disposal was preceded by an increase in FOX starting at 90 min during the LIPID clamp (CON 6.7 ± 0.0 vs. LIPID 9.3 ± 0.8 $\mu\text{mol/kg lean mass/min}$, $P < 0.05$, Fig 5.3C) and later by a reduction in COX, which began at 150 min (CON 23.9 ± 0.0 vs. LIPID 17.8 ± 1.0 , $P < 0.05$, Fig 5.3B). From these time points onwards, both FOX and COX continued to increase and decrease, respectively, until beginning to plateau at the end of the clamp at 360 min (Fig 5.3B and 5.3C). At the end of the clamp, FOX was over 2.5 fold higher (CON 4.9 ± 0.0 vs. LIPID 12.8 ± 0.7 $\mu\text{mol/kg lean mass/min}$, $P < 0.001$, Fig 5.3C) and COX was over 3 fold lower (CON 23.4 ± 0.0 vs. LIPID 7.6 ± 1.6 $\mu\text{mol/kg lean mass/min}$, $P < 0.001$, Fig 5.3B) in the LIPID than the CON trial.

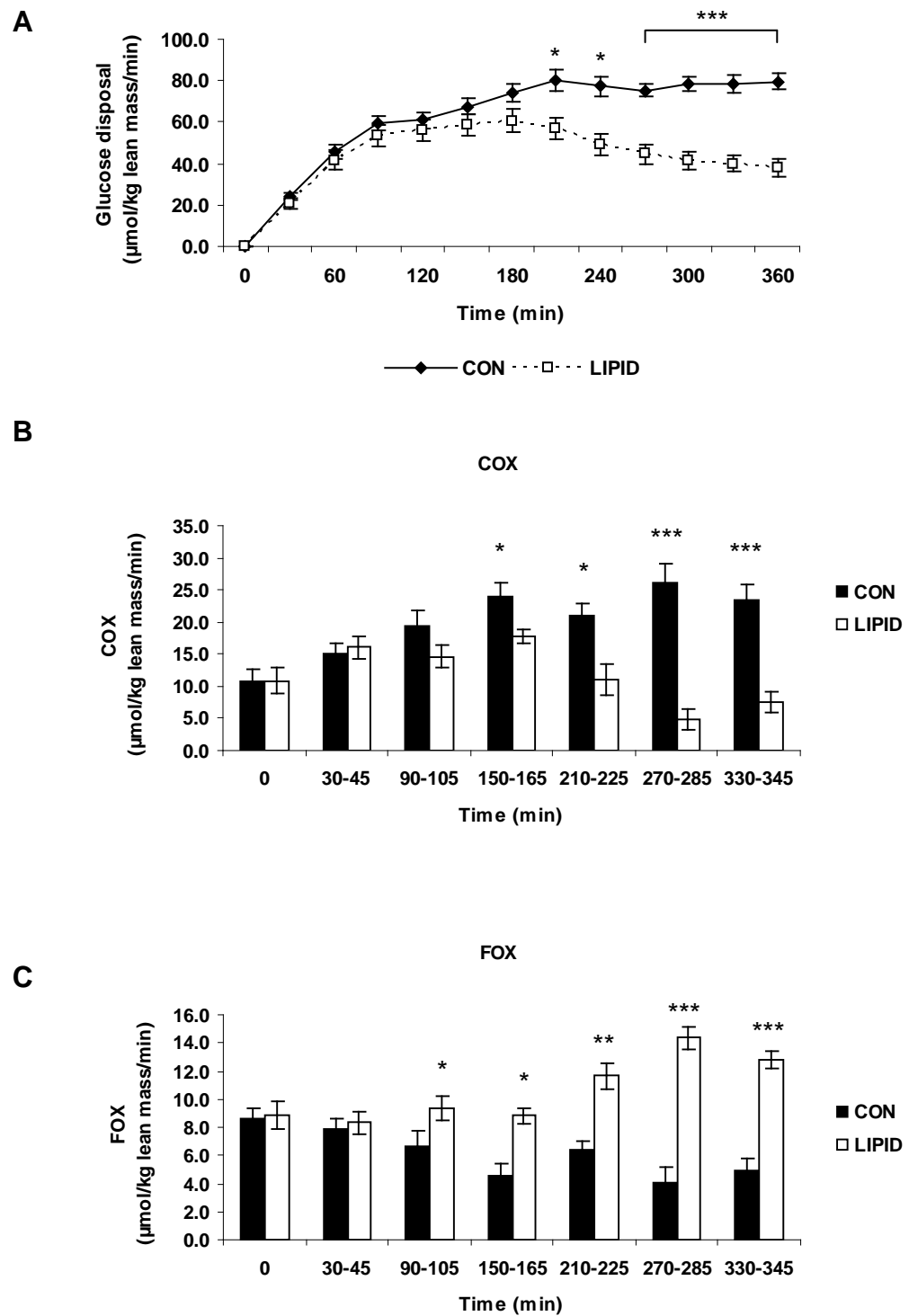


Figure 5.3 Glucose disposal (A), carbohydrate oxidation (COX) (B) and fat oxidation (FOX) (C) during the insulin clamp with (LIPID) or without (CON) the simultaneous infusion of Intralipid (n = 10).

*P<0.05, **P<0.01, ***P<0.001 vs. corresponding CON/LIPID value

5.3.3 Calpain-10 and GLUT4 expression

Calpain-10 mRNA was significantly reduced in both CON (PRE CLAMP 0.93 ± 0.08 vs. 6 h POST CLAMP 0.68 ± 0.06) and LIPID (PRE CLAMP 0.87 ± 0.12 vs. 6 h POST CLAMP 0.66 ± 0.02) trials after 6 h ($P < 0.05$) of insulin infusion (mixed model analysis: insulin effect, $P = 0.016$), and the addition of Intralipid in the LIPID trial did not modify this response (Fig 5.4A).

To more clearly differentiate the effect of insulin and lipid on calpain-10 mRNA expression, a separate group of subjects ($n=6$) were infused with Intralipid infusion alone for 3 h in the absence of insulin. Consistent with the findings above, Intralipid infusion alone had no effect on calpain-10 expression (Fig 5.6).

Protein levels of full length 75 kDa calpain-10 were not different with insulin infusion in either the CON (PRE CLAMP 0.64 ± 0.13 vs. 3 h POST CLAMP 0.58 ± 0.10 vs. 6 h POST CLAMP 0.55 ± 0.09) or LIPID trials (PRE CLAMP 0.46 ± 0.09 vs. 3 h POST CLAMP 0.39 ± 0.08 vs. 6 h POST CLAMP 0.43 ± 0.07) (Fig 5.5). There was a borderline significant effect of the LIPID treatment on calpain-10 protein levels ($P=0.05$) but this was most likely due to the slightly lower values prior to the commencement of the infusions (Fig 5.5). In terms of calpain-10 isoforms, additional immunoreactive bands were observed at

approximately 60, 48 and 35 kDa in the supernatant fraction using the new extraction buffer (Fig 5.5).

Measurement of GLUT4 mRNA revealed no significant effect of insulin (PRE CLAMP 1.2 ± 0.1 vs. 3 h POST CLAMP 1.2 ± 0.1 vs. 6 h POST CLAMP 1.2 ± 0.1) or insulin and Intralipid infusion (PRE CLAMP 1.2 ± 0.1 vs. 3 h POST CLAMP 1.1 ± 0.1 vs. 6 h POST CLAMP 1.1 ± 0.1) on skeletal GLUT4 mRNA levels (Fig 5.4B).

5.3.4 Calpain-3 expression

Calpain-3 mRNA was not affected by insulin infusion alone in the CON trial (PRE CLAMP 2.3 ± 0.2 vs. 6 h POST CLAMP 2.0 ± 0.2) or by intralipid and insulin infusion in the LIPID trial (PRE CLAMP 2.1 ± 0.1 vs. 6 h POST CLAMP 2.0 ± 0.1) (Fig 5.6).

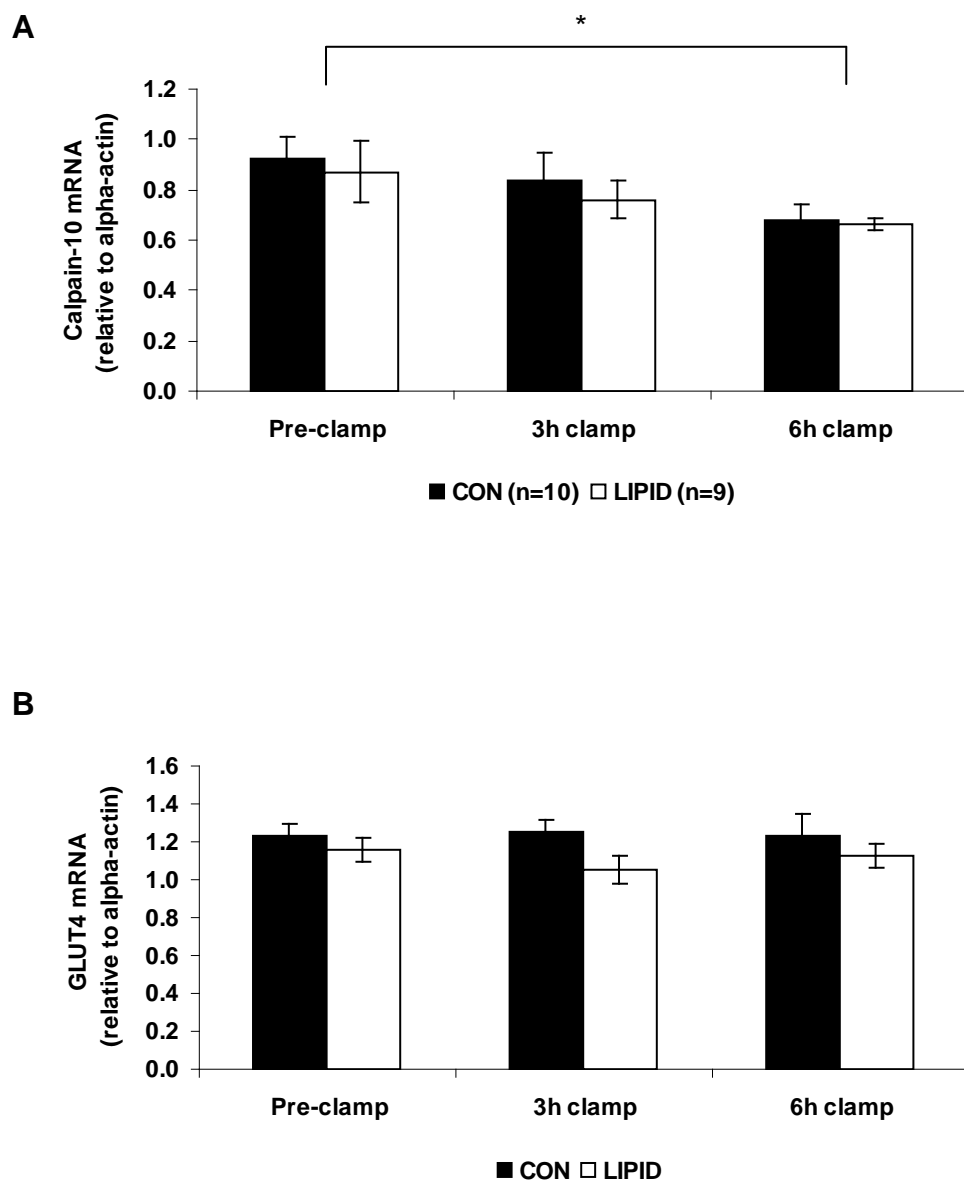


Figure 5.4 The effect of insulin infusion with or without Intralipid infusion on skeletal muscle calpain-10 (A) and GLUT4 (B) mRNA levels (n = 10).

* $P < 0.05$ effect of insulin infusion on calpain-10 mRNA levels in both CON and LIPID trials.

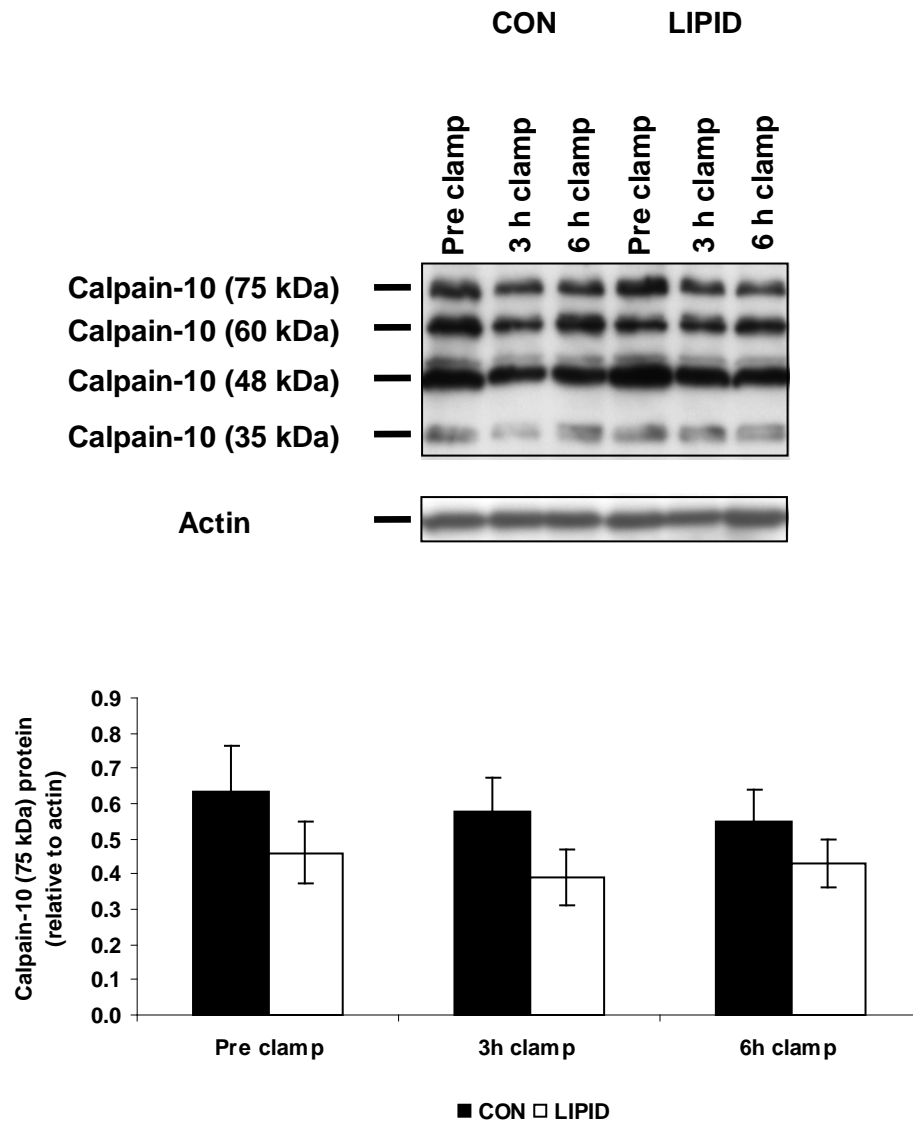


Figure 5.5 The effect of insulin infusion with or without Intralipid infusion on skeletal muscle full length calpain-10 (n = 10).

Representative blots showing all observed calpain-10 immunoreactive bands in addition to the control protein (actin, 45 kDa). Only the results for the full length calpain-10 protein (75 kDa) are shown. Calpain-10 and actin were analysed on the same Western blot.

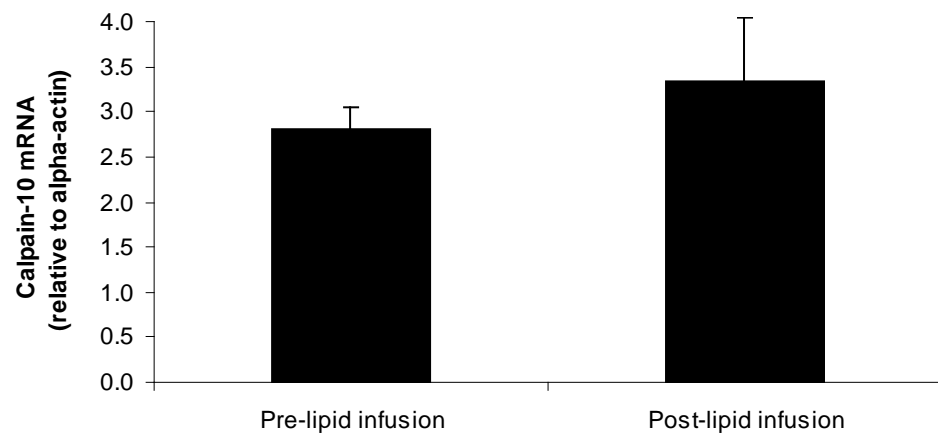


Figure 5.6 The effect of Intralipid infusion alone for 3 h on calpain-10 mRNA expression (n=6).

5.4 Discussion

The main finding from this chapter is that 6 h of insulin infusion significantly downregulated calpain-10 mRNA. The addition of Intralipid to the insulin clamp did not modify the response of calpain-10 to insulin infusion despite a significant reduction in glucose disposal 3 h after the commencement of the Intralipid infusion. Infusion of Intralipid alone in a separate group of subjects for 3 h did not affect calpain-10 expression, strengthening the finding that insulin and not Intralipid was responsible for the reduction in calpain-10 mRNA in the present study. GLUT4 mRNA levels were not affected by either insulin or Intralipid and insulin infusion. These data suggest that calpain-10 may be regulated by hyperinsulinaemia *per se* and is not influenced by changes in insulin mediated glucose uptake. This is interesting since no previous studies have investigated the effect of insulin infusion on skeletal muscle calpain-10 expression. Moreover, in the previous studies in this thesis, the effect of insulin on calpain-10 was not investigated nor controlled for. Whilst in chapter 4 a small trend for reduced calpain-10 protein expression was observed with 4 h of insulin infusion following both CON and HF diets, it is not clear what impact each diet had on this response.

Intralipid infusion has been shown to reduce insulin mediated glucose disposal in humans and previous studies have indicated that the skeletal muscle is the primary site of the fatty acid mediated insulin resistance (Ferrannini et al., 1983). More recent studies have shown

that this insulin resistance is associated with impairments in insulin receptor and insulin receptor substrate (IRS-1) tyrosine phosphorylation, phosphatidylinositol 3-kinase (PI3K) activity associated with IRS1 and AKT/PKB serine phosphorylation in skeletal muscle (Belfort et al., 2005; Dresner et al., 1999; Kruszynska et al., 2002) indicating significant impairments in the insulin signalling pathway with Intralipid infusion in humans.

Carlsson et al. (2005) were the first group to investigate the effect of insulin and insulin plus Intralipid infusion on skeletal muscle calpain-10 mRNA levels. In this study the response of calpain-10 mRNA to 2 h of insulin infusion following lipid infusion for 0, 2 and 24 h in normal glucose tolerant (NGT) and impaired glucose intolerant (IGT) individuals was investigated. Firstly, a 2 h insulin infusion alone had no effect on calpain-10 mRNA levels in NGT or IGT group. However, following 24 h of Intralipid infusion, calpain-10 mRNA was increased 3-fold in response to 2 h of insulin infusion in the NGT but not the IGT subjects. The data in the current study do not confirm the findings of Carlsson et al. (2005) as 1) insulin infusion alone downregulated calpain-10 mRNA expression and 2) the addition of lipid to the insulin clamp procedure did not affect the response of calpain-10 mRNA to insulin infusion alone. This was despite significant reductions in glucose disposal and alterations in COX and FOX with the addition of lipid to the insulin clamp. Extending the work of Carlsson et al. (2005) further to include the analysis of calpain-10 protein levels revealed that calpain-10 protein

levels were not affected by insulin infusion or by intralipid and insulin infusion.

The possible explanation for these discrepancies between the present study and that of Carlsson et al. (2005) are not entirely clear but could be due to a number of factors. Most importantly, however, the study protocols were significantly different. For example in the present study, lipid and insulin were infused together for the same period of time (6 h) whereas Carlsson and co-authors infused Intralipid alone for the required times (up to 24 h) and then performed a 2 h insulin clamp whilst the lipid infusion continued. Infusion of lipid alone for 24 h might be expected to profoundly affect the subsequent physiological response to insulin infusion and therefore may substantially alter the effect of insulin on calpain-10 mRNA.

Carlsson et al. (2005) suggested that the increase in calpain-10 mRNA in the NGT but not the IGT group with insulin following lipid infusion may be related to an interaction between calpain-10 and GLUT4 and on the involvement of calpain-10 in a novel apoptosis pathway originally identified in pancreatic β -cells and which may be initiated by the fatty acid palmitate (Johnson et al., 2004). Whilst it is not possible to rule out any potential interaction between GLUT4 and calpain-10 post-translationally, GLUT4 mRNA was unchanged by lipid and insulin infusion in the present study.

It is not clear how a calpain-10 mediated ordered lysis of muscle cells, possibly initiated by palmitate, would protect NGT but not IGT subjects from FFA mediated insulin resistance. Palmitate has been shown to induce insulin resistance in myotubes *in vitro* (Storz et al., 1999) but has not been linked to apoptosis in muscle cells (Johnson et al., 2004). Regardless it is not possible to reach the conclusion that increased calpain-10 expression *per se* protects subjects from FFA mediated insulin resistance in the present study as glucose disposal was significantly downregulated after 210 min of the clamp and was 50% lower at the end of the clamp when lipid was present. This occurred without any change in calpain-10 mRNA or protein levels and it therefore seems unlikely that calpain-10 expression plays any role in modifying the physiological response to FFA. Interestingly, no data was reported on the effect of the increase in calpain-10 on insulin mediated glucose disposal in the study by Carlsson et al. (2005). If a protective role for calpain-10 was to be envisaged in this pathway, one hypothesis could be that increased calpain-10 activity (not expression) would attenuate the protein kinase C (PKC) mediated insulin resistance in response to elevated FFA (Itani et al., 2002).

The observation that calpain-10 mRNA but not protein expression was significantly downregulated by 6 h insulin infusion in both trials suggests the possibility that calpain-10 expression is regulated by hyperinsulinaemia. In **chapter 4**, 4 h of insulin infusion tended to lead to a reduction in calpain-10 protein. This finding is consistent with the

present study as calpain-10 mRNA was only reduced at the end of the clamp (6 h) and was no different between 0 h and 3 h of the clamp. This observation also provides an additional explanation as to why Carlsson et al. (2005) did not see any effect of a 2 h insulin infusion on calpain-10 mRNA levels. It seems likely that the reduction in calpain-10 mRNA was indeed the result of the hyperinsulinaemia and was not important for the increase in glucose uptake during the insulin infusions as the reduction was seen in both arms of the trial. This is strengthened because the change in calpain-10 occurred only towards the end of the 6 h clamp and a similar effect was not seen with only 4 h of insulin infusion (**Chapter 4**). Moreover, lipid infusion alone in the absence of insulin had no effect on calpain-10 expression in the present study. What is particularly interesting is that a reduction in calpain-10 mRNA with hyperinsulinaemia might provide a link between calpain-10 and diabetes. Chronic hyperinsulinaemia, which is characteristic of some insulin resistant states, may lead to a similar reduction in calpain-10 mRNA levels in skeletal muscle. Because calpain-10 protein levels were unaffected by insulin infusion it is unclear what impact, if any, the observed change in calpain-10 mRNA would have on skeletal muscle metabolism. Further studies in human subjects with long-term hyperinsulinaemia are needed to clarify the role of insulin on the expression of calpain-10 in skeletal muscle.

A number of immunoreactive bands were observed in the supernatant fraction for calpain-10 following extraction with the new protein

extraction buffer (see **general methods, 2.2.7.6**). The intensity of all of these bands was unchanged with insulin infusion, however, and this is consistent with the findings from chapter 4, where 4 h of insulin infusion had no effect on calpain-10 protein levels. The impact of the determination of calpain-10 protein in the supernatant fraction on the interpretation of the data and on comparisons with earlier chapters of this thesis is likely to be minimal as it was shown in chapter 2 that when using buffer A and B, the majority of calpain-10 is detected in the pellet and supernatant fractions, respectively, indicating that in both cases the majority of calpain-10 protein is detected.

5.5 Conclusions

The major finding in the present study is that 6 h of insulin infusion downregulated calpain-10 mRNA in human skeletal muscle. This effect of insulin on calpain-10 was not altered by the simultaneous infusion of Intralipid – which lead to marked reductions in insulin mediated glucose uptake and COX and a large increase in FOX. Moreover, Intralipid infusion alone had no effect on calpain-10 suggesting that hyperinsulinaemia *per se* regulates calpain-10 mRNA expression. There was no effect of insulin on calpain-10 protein levels and the impact of the observed reduction in calpain-10 mRNA with insulin on skeletal muscle metabolism is therefore unclear. Further studies examining the long-term effect of hyperinsulinaemia on calpain-10 mRNA and protein expression in skeletal muscle will be needed to more

clearly define the role of insulin mediated regulation of calpain-10 and its relationship to the development of insulin resistance and type 2 diabetes.

6 Exercise mediated glucose uptake and the expression of genes involved in glucose transport and oxidation

6.1 Introduction

The expression of calpain-10 and -3 was not altered in induced states of insulin resistance in healthy humans as shown in the previous chapters of this thesis. This chapter, therefore, set out to investigate the effect of an increase in insulin sensitivity on the expression of these genes. A single bout of moderate intensity exercise has been shown to lead to significant improvements in skeletal muscle insulin sensitivity and glucose uptake (e.g., Mikines et al., 1988; Richter et al., 1989) and this effect can last for up to 48 h post exercise bout (Mikines et al., 1988). The precise mechanisms responsible for 1) the immediate effect of exercise on muscle glucose uptake and 2) the increase in insulin stimulated glucose uptake during recovery from exercise are not well understood. During exercise calcium induced activation of calcium-calmodulin-dependent protein kinase (CaMKII) (Wright et al., 2004) and/or AMP-activated protein kinase (AMPK) (Winder and Hardie, 1996) is thought to be responsible for the contraction mediated increase in the translocation of GLUT4 in an insulin independent fashion (Ploug et al., 1984).

Following exercise, the exact mechanisms responsible for the enhancement in insulin mediated glucose uptake are unknown. One hypothesis is that exercise potentiates the insulin signalling pathway. Immediately after exercise *in vivo*, insulin mediated activity of IRS-2 associated with PI3K is increased in the exercise leg versus the control leg (Howlett et al., 2002). It has been suggested that this could lead to an increase in aPKC mediated GLUT4 translocation (Wojtaszewski and Richter, 2006). This potential enhancement in insulin signalling pathways following exercise could be linked to the glycogen lowering effect of exercise; reduced glycogen levels have been shown to activate GS (Nielson et al., 2001). Moreover, prior exercise is known to increase GS activity in response to stimulation by insulin (Wojtaszewski et al., 2000). This in turn could be related to the effect of glycogen on the insulin stimulated activation of AKT/PKB as insulin mediated AKT/PKB activity is enhanced by lowering glycogen levels (Derave et al., 2000).

The increase in insulin sensitivity following a bout of exercise could also be the result of adaptive changes in the expression of genes important in the regulation of glucose transport into skeletal muscle. Some studies have suggested that GLUT4 mRNA is increased immediately after exercise (Kraniou et al., 2006) whilst others have demonstrated no effect of exercise on the GLUT4 message (Vissing et al., 2005). The hypothesised link between calpain-10 and GLUT4 trafficking has been discussed previously in this thesis and this interaction, combined with

the elevated intracellular calcium concentrations during exercise, provides a potential mechanisms whereby calpain-10 may contribute to exercise mediated GLUT4 translocation. It is possible that an increase in insulin sensitivity with exercise will be associated with an increase in calpain-10 expression. An inhibition of general calpain expression and activity in calpastatin overexpressing mice leads to an increase in CaMKII and AMPK expression (Otani et al., 2006) and it is possible that calpain-10 mediates an increase in insulin sensitivity following exercise via these pathways. Calpain-3 expression following both eccentric and concentric exercise bouts in humans has previously been investigated. Muscular damage as a result of 30 min of downhill running was associated with a decrease in skeletal muscle calpain-3 mRNA (Feasson et al., 2002), whilst no significant autolysis of calpain-3 was observed with exhaustive bicycling exercise (Murphy et al., 2006).

Adaptive changes in other genes important for glucose phosphorylation and oxidation in skeletal muscle may also be important for the increase in insulin sensitivity following exercise. For example, during recovery from a single bout of exercise, the transcriptional activity and mRNA content of PDK4 is increased (Pilegaard et al., 2000), although this effect was not apparent 24 h following the exercise bout. Similarly, the expression of HKII has been shown to be upregulated during recovery from exercise in humans (Pilegaard et al., 2005) and rats (O'Doherty et al., 1994). The peroxisome proliferator-activated receptor- γ (PPAR γ) coactivator 1 α (PGC1 α) has been linked to transcriptional regulation of

GLUT4 and therefore has also been associated with the metabolic adaptation to exercise. For example, in rats, low intensity swimming exercise leads to an increase in PGC1 α expression (Terada et al., 2002), and similar results have been found in humans (Pilegaard et al., 2003b).

The aim of the present chapter was to investigate the effects of a single bout of moderate intensity one-legged cycling exercise, followed by an insulin clamp 24 h later, on skeletal muscle gene expression in order to gain a better understanding of the metabolic adaptations to a single bout of exercise in humans. To achieve this aim, the expression of genes potentially important for glucose uptake (GLUT4, calpain-10 and PGC1 α) and oxidation/phosphorylation (HKII, PDK4 and PDK2) were determined. It was hypothesised that an increase in insulin sensitivity as a result of exercise would be associated with alterations in the expression of all of these genes. In particular, as the previous chapter demonstrated that calpain-10 was decreased with insulin infusion, it was hypothesised that exercise would increase calpain-10 expression (with increased insulin sensitivity) and that insulin infusion following exercise would augment this response.

6.2 Subjects and Methods

6.2.1 Study protocol

Eight healthy men (age 24 ± 2 yr, body mass 79 ± 4 kg, BMI 24 ± 1 kg.m²) were recruited from the students of the University of Nottingham as described previously.

The study protocol is summarised in Fig 6.1. Each individual underwent a 4 h hyperinsulinaemic-euglycaemic clamp 24 h after completing 90 min of one-legged moderate intensity (60% of maximal oxygen uptake) exercise. Prior to the commencement of the study, subjects were asked to complete an incremental exercise test on a cycle ergometer to establish their two-legged $\dot{V}O_2$ max as described in the **general methods (2.2.5)**. The corresponding one-legged $\dot{V}O_2$ max was calculated as 75% of the two-legged value, as previously described (Pernow and Saltin, 1971). Linear regression was then used to estimate the work load required for a given percentage of the $\dot{V}O_2$ max.

On day 1 of the study, subjects reported to the laboratory at approximately 8 am after an overnight fast and a catheter was placed into a dorsal hand vein in a retrograde fashion and placed in heated chamber at 55°C to obtain arterialized blood samples as described

previously (**general methods, 2.2.2**). To ensure that the first post exercise biopsy was obtained as quickly as possible after the cessation of exercise, the biopsy site on the leg to be exercised (EX) was prepared and anaesthetised prior to the exercise bout as described in the **general methods (2.2.7)**. Subjects were then asked to perform the 90 min of one-legged exercise on the cycle ergometer. Briefly, two stationary exercise bicycles were placed either side of an electrically braked cycle ergometer and the seats were adjusted to allow for comfortable peddling of the ergometer whilst the subject remained seated on the stationary bicycle. The non-exercising leg (CON) was rested on the stationary bicycle and care was taken to ensure that this leg remained as inactive as possible. The opposing side of the ergometer was peddled by the same helper on all occasions. Four subjects exercised with their left leg and the remaining four with their right leg. Heart rate monitors were placed on each participant to monitor workload throughout the exercise period and oxygen consumption measurements were taken every 30 min to ensure that the subject was performing the work required. Blood samples were also taken at 15 min intervals during the exercise period. Following the 90 min of exercise, the subject was quickly moved to the bed and placed in a supine position where the first biopsy was taken from the EX leg and then the CON leg, which was used as the baseline sample. The subject was then fed a mixed meal and was prescribed additional mixed meals (55% CHO, 30% fat, 15% protein) to consume for the remainder of the 24 h

recovery period, which was based on the subjects usual dietary habits which were obtained for three days prior to the study using food diaries.

On day 2, subjects reported to the laboratory at approximately 8 am after an overnight fast and after baseline measurements were made, muscle biopsies were taken from both the CON and EX leg, as described previously (**general methods, 2.2.7**). A standard 4 h hyperinsulinaemic-euglycaemic clamp was then started at approximately 9 am as described in the **general methods (2.2.6)**. Determinations of substrate oxidation rate were obtained before and at steady state during the last 30 min the insulin clamp.

6.2.2 Blood and urine analysis

Collected blood samples were analysed for blood glucose, serum insulin and plasma FFA as described under the **general methods (2.2.2)** section.

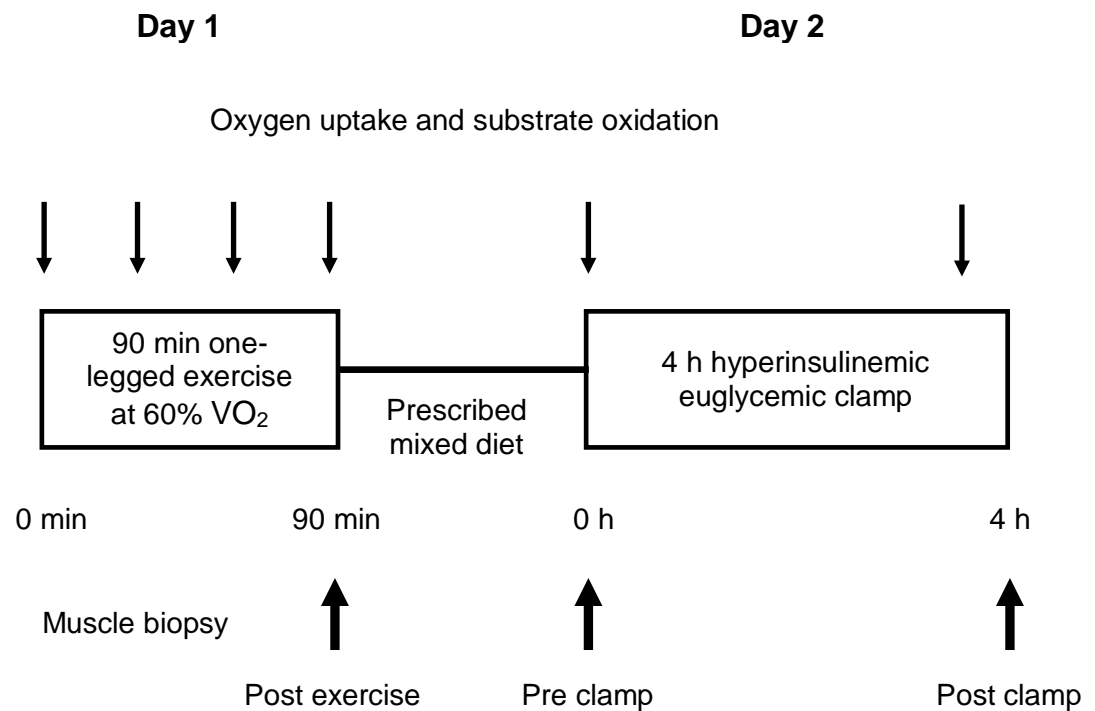


Figure 6.1 Schematic representation of the exercise study protocol.

Skeletal muscle biopsies were taken immediately following the exercise bout in the CON and EX leg on day 1, and before and after the insulin clamp 24 h later on day 2. Estimates of substrate oxidation were taken at 30 min intervals during the exercise protocol on day 1 and before and at the end of the insulin clamp on day 2.

6.2.3 Substrate oxidation rates

Rates of carbohydrate (COX) and fat oxidation (FOX) were calculated prior to the exercise on day 1, and before and at steady state during the insulin clamp, from the $\dot{V}CO_2$ and $\dot{V}O_2$ measurements which were obtained using the open-circuit indirect calorimeter, as described in the **general methods (2.2.4)**. During the exercise period, rates of oxidation were similarly determined from the $\dot{V}CO_2$ and $\dot{V}O_2$ measurements obtained using an alternative machine (Vmax, Sensor Medics, Yorba Linda, CA) which was attached to a mouthpiece and valve.

6.2.4 Whole body glucose disposal calculations

Whole body glucose disposal values were calculated from the glucose infusion rate (GIR) during the clamp on day 2 as described in **chapter 4 (4.2.6)**.

6.2.5 Skeletal muscle biopsy analysis

Skeletal muscle biopsies were analysed for calpain-10 and -3, GLUT4, PDK2, PDK4, HKII and PGC1 α mRNA expression using RT-PCR, as described in the **general methods (2.2.7)**.

6.2.6 Statistics

Data were analysed as described in the **general methods (2.6)**.

6.3 Results

6.3.1 Blood metabolites

Exercise decreased circulating insulin (PRE EX 7.1 ± 0.8 vs. 90 min EX 3.6 ± 0.4 mU/L, $P < 0.01$) (Fig 6.2A) and increased plasma FFA (PRE EX 0.3 ± 0.1 vs. 90 min EX 0.7 ± 0.1 mmol/L, $P < 0.05$) (Fig 6.2B). These values returned to their pre exercise values prior to the insulin clamp on day 2 as shown in Fig 6.2. During the insulin clamp on day 2 physiological serum insulin concentrations were achieved (78.0 ± 6.0 mU/L) and plasma FFA were suppressed (PRE CLAMP 0.3 ± 0.04 vs. 240 min CLAMP 0.02 ± 0.004 mmol/L) at the end of the clamp period during steady state.

6.3.2 Substrate oxidation

Carbohydrate oxidation was elevated 30 min into the exercise period (PRE EX 11.6 ± 1.5 vs. 30 MIN EX 94.8 ± 10.2 $\mu\text{mol/kg/min}$, $P < 0.001$) and remained at this level until the exercise had ceased (Fig 6.2C). Similarly FOX increased after 30 min of exercise (PRE EX 5.5 ± 0.7 vs. 30 MIN EX 28.3 ± 4.5 $\mu\text{mol/kg/min}$, $P < 0.01$) and remained essentially unchanged until the exercise period was over (Fig 6.2C). On the morning of day 2, both COX (13.1 ± 0.8 $\mu\text{mol/kg/min}$) and FOX (5.6 ± 0.5 $\mu\text{mol/kg/min}$) had returned to their pre exercise values (Fig 6.2C).

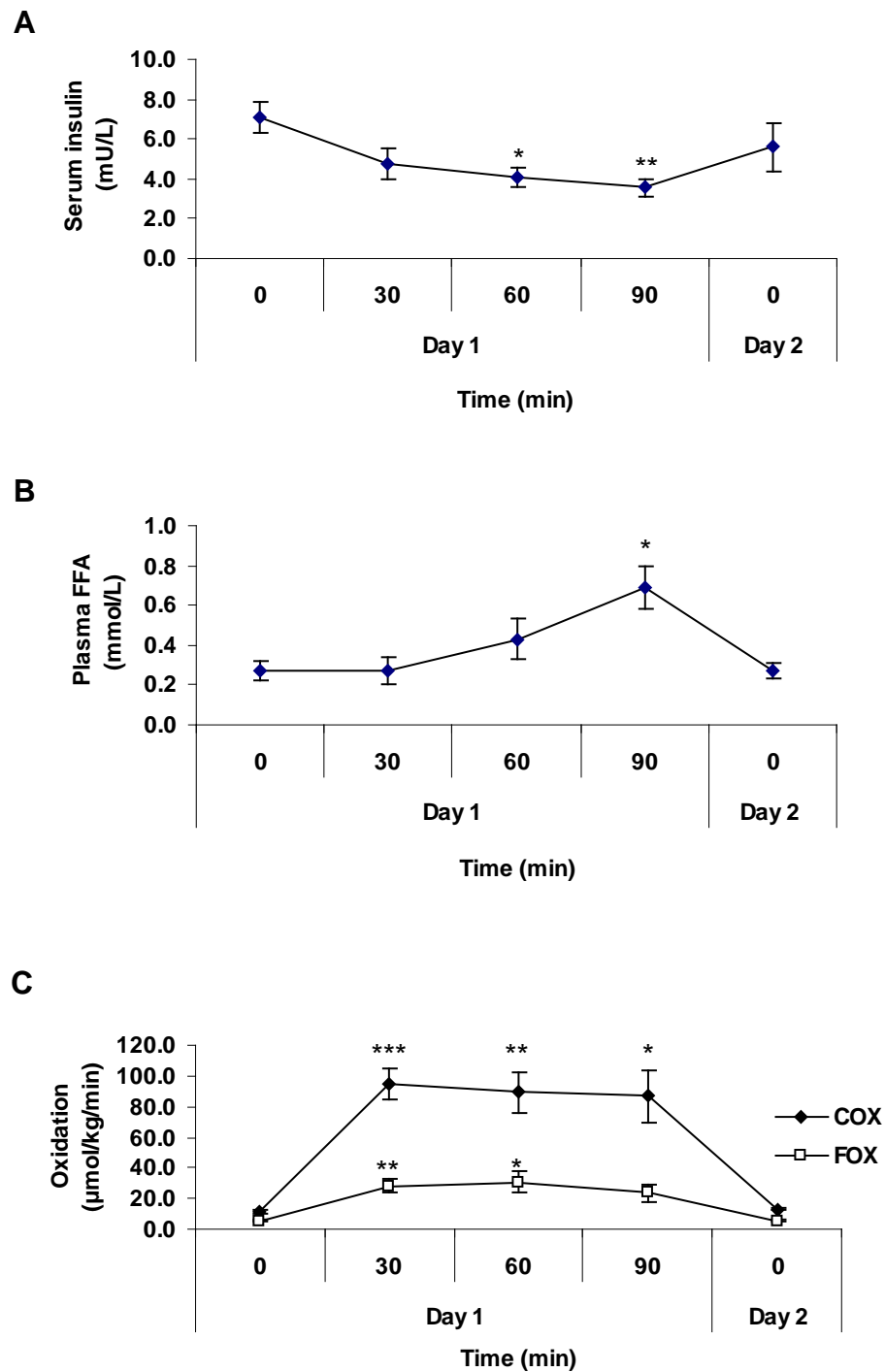


Figure 6.2 Serum insulin (A), plasma FFA (B) and substrate oxidation rates (C) before and during (day 1) and 24 h after (day 2) 90 min of one-legged exercise (n = 8).

*P<0.05, **P<0.01, ***P<0.001 vs. pre exercise value

6.3.3 Gene expression

The mRNA expression of calpain-10 and -3 and GLUT4 were not different between the EX and CON leg at any time point (Fig 6.4). The mRNA levels of PDK4 were also investigated in this chapter and the expression of PDK4 was significantly reduced immediately after exercise in the EX leg (POST EX 1.4 ± 0.3) versus the CON leg (POST EX 2.1 ± 0.4) ($P < 0.05$) (Fig 6.5A). In contrast, mRNA levels of PDK2 were not altered immediately after exercise (POST EX (CON) 0.7 ± 0.1 vs. POST EX (EX) 0.8 ± 0.03) (Fig 6.5B). 24 h after the exercise period, PDK4 mRNA levels were significantly reduced in the CON leg (POST EX 2.1 ± 0.4 vs. 24 h POST EX 1.0 ± 0.2 , $P < 0.05$), and were almost identical to the EX leg (24 h POST EX 1.0 ± 0.2). PDK2 mRNA was higher in the EX (24 h POST EX 0.9 ± 0.1) but not the CON (24 h POST EX 0.7 ± 0.04) leg 24 h after exercise ($P < 0.01$). Insulin infusion reduced PDK4 mRNA further to the same extent in both legs (POST CLAMP (CON) 0.2 ± 0.03 vs. POST CLAMP (EX) 0.2 ± 0.03 , $P < 0.01$), whereas insulin did not have any additional effect on PDK2 mRNA levels (Fig 6.5B).

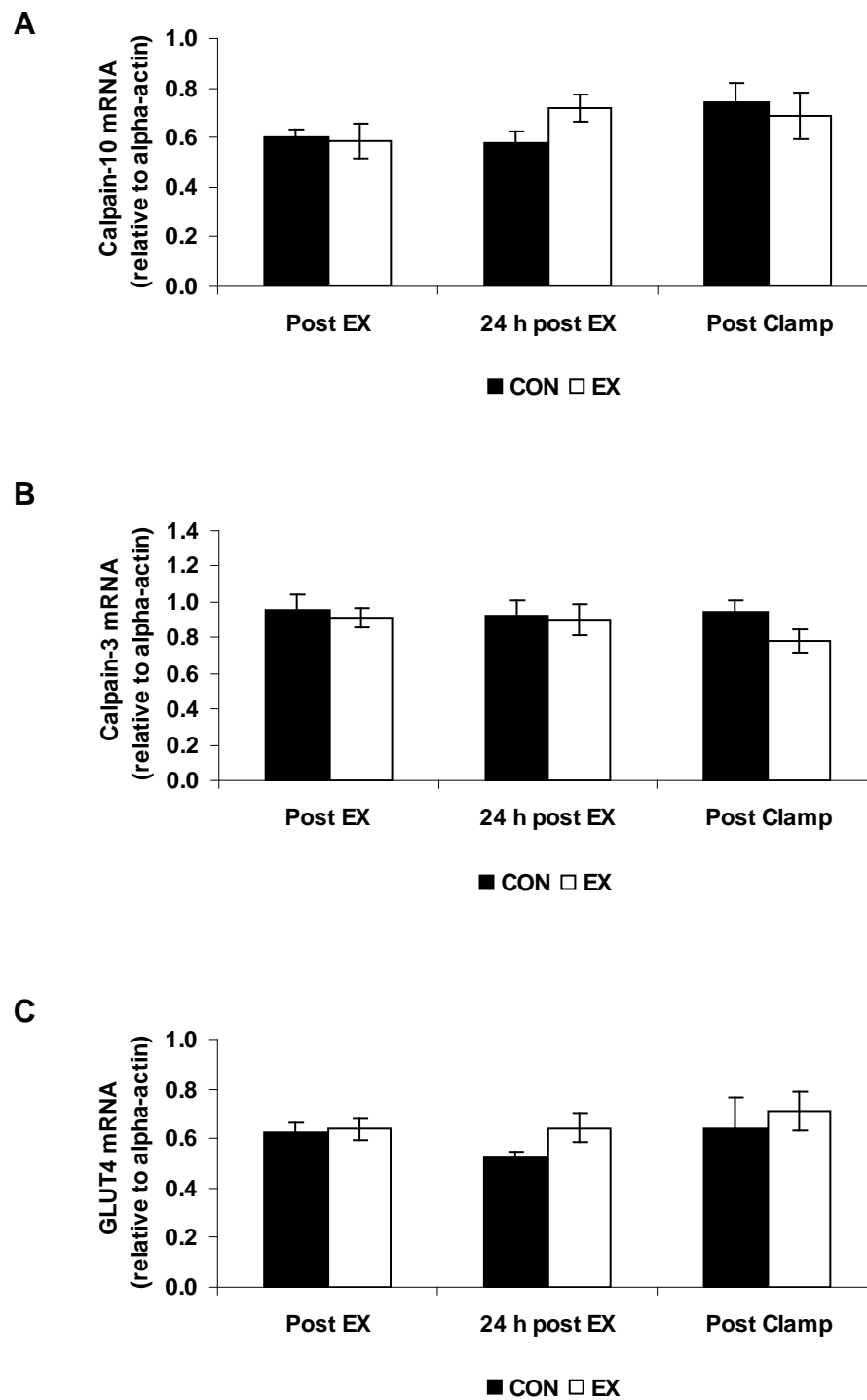
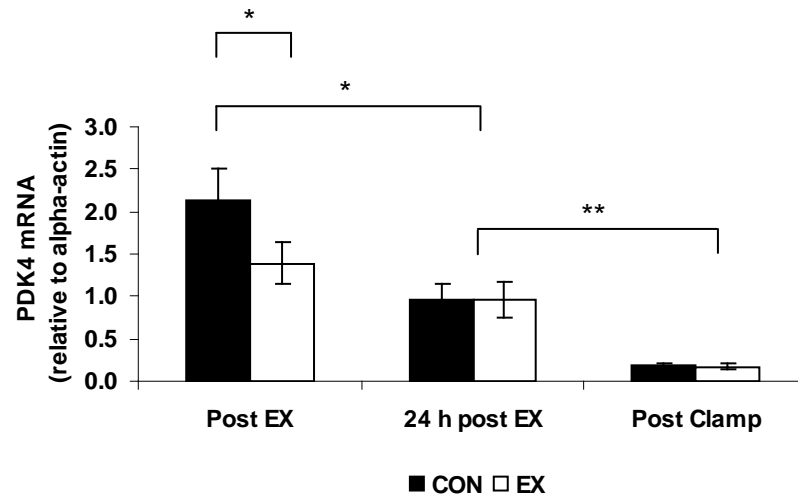


Figure 6.3 The effect of a 90 min one-legged exercise bout on calpain-10 (A) and calpain-3 (B) and GLUT4 (C) mRNA immediately after and 24 h post exercise and following a 4 h insulin clamp (n = 8).

A



B

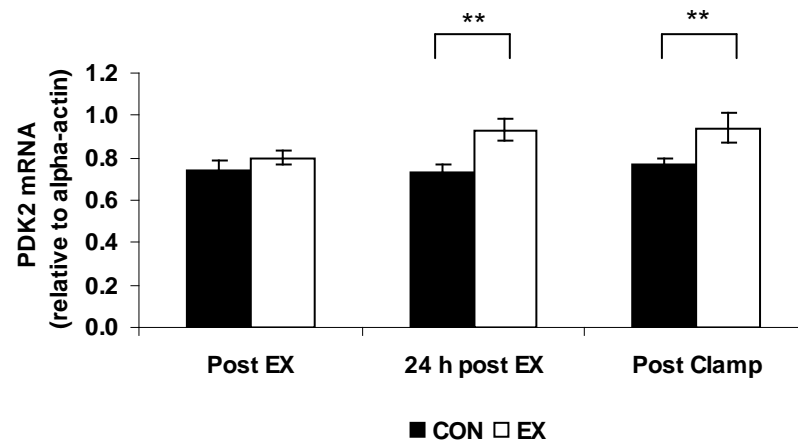
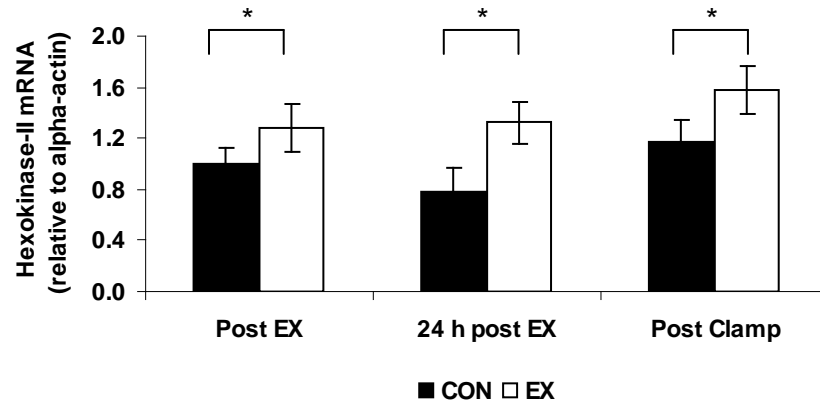


Figure 6.4 The effect of a 90 min one-legged exercise bout on PDK4 (A) and PDK2 (B) mRNA immediately after and 24 h post exercise and following a 4 h insulin clamp (n = 8).

*P<0.05, **P<0.01 vs. previous time point or CON value as indicated

The expression of hexokinase-II was similarly increased in the EX leg immediately following exercise (POST EX (CON) 1.0 ± 0.1 vs. POST EX (EX) 1.3 ± 0.2 , $P < 0.05$) and 24 h after the exercise bout (24 h POST EX (CON) 0.8 ± 0.2 vs. 24 h POST EX (EX) 1.3 ± 0.2 , $P < 0.05$), and was also higher in the EX leg following insulin infusion ($P < 0.05$) (Fig 6.6A). The mRNA levels of PGC1 α were significantly elevated in the exercise leg 24 h following the exercise bout when compared to the control leg ($P < 0.05$) (Fig 6.6B).

A



B

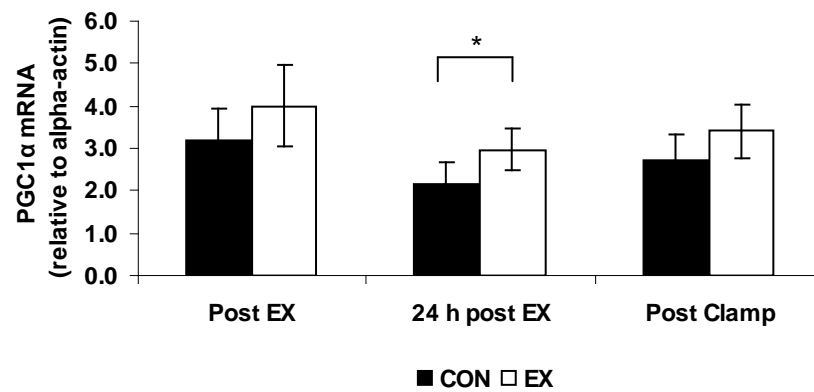


Figure 6.5 The effect of a 90 min one-legged exercise bout on HKII (A) and PGC1α (B) mRNA immediately after and 24 h post exercise and following a 4 h insulin clamp (n = 8).

*P < 0.05 vs. CON leg value

6.4 Discussion

The present study investigated the effect of a single bout of exercise on the expression of genes thought important for glucose transport and oxidation/phosphorylation using a one-legged cycling model. Gene expression was measured immediately following and 24 h after cycling exercise under basal and insulin stimulated conditions during a 4 h hyperinsulinaemic-euglycaemic clamp. The main findings from this chapter are that an exercise mediated increase in glucose uptake is not associated with changes in the mRNA expression of genes believed to be important for glucose transport (calpain-10 and GLUT4), but is associated with significant alterations in genes that are important for glucose oxidation (PDK2 and PDK4) and phosphorylation (HKII).

Calpains and exercise

In the previous chapter of this thesis (**chapters 5**), insulin sensitivity and glucose uptake into skeletal muscle was altered in a negative fashion via an increase in FFA availability. The results from this study indicated that FFA mediated insulin resistance was not associated with any significant changes in calpain-10 mRNA and protein expression in skeletal muscle. However, it was also shown that hyperinsulinaemia negatively regulated calpain-10 mRNA. In this chapter, the ability of exercise to regulate calpain-10 expression was tested. It was hypothesised that an exercise mediated increase in insulin sensitivity

and glucose uptake both during and 24 h after exercise might be associated with an adaptive increase in calpain expression. Moreover, calpain-3 is intricately linked to skeletal muscle via interaction with titin, a giant protein which spans the muscle half-sarcomere from the M to the Z line (Sorimachi et al., 1995). Previous studies have implicated a role for calpain-3 during eccentric muscle contraction (Feasson et al., 2002) and the present study examined whether a single bout of exercise leads to changes in the expression of calpain-3 mRNA in humans.

Exercise leads to a significant increase in glucose uptake into skeletal muscle which has been shown to occur independently of insulin (Ploug et al., 1984; Richter et al., 1985; Wallberg-Henriksson and Holloszy, 1985). The translocation of GLUT4 to the plasma membrane is increased with exercise via as yet unknown mechanisms, but is thought to occur via calcium release from the sarcoplasmic reticulum resulting in the activation of Ca^{2+} /calmodulin-dependent protein kinase (CaMKII) and/or an AMPK related mechanism linked to the energy status of the cell (see Wojtaszewski and Richter, 2006 for review). Whilst the downstream mechanisms linking increased calcium release to GLUT4 translocation are currently unknown, it was hypothesised in this chapter that calpains may be important in this putative pathway. This may be especially true given the proposed link between calpain-10 and GLUT4 in skeletal muscle and that calpains are activated in states of elevated intracellular calcium concentrations, such as those seen during

exercise. A potential link between calpain-10 and GLUT4 translocation during exercise involving calcium is CaMKII and this has been shown to be increased markedly following calpain inhibition in rodents (Otani et al., 2006). Changes in calpain-10 expression during exercise may mediate an increase in GLUT4 translocation via CaMKII. However, in the present study, exercise had no effect on calpain-10 expression suggesting that this pathway, at least at the level of mRNA, is not important during muscle contraction. Indeed, this is consistent with the studies of Otani et al. (2006) as they reported no change in contraction mediated glucose uptake or GLUT4 translocation following calpain inhibition despite the observed increase in CaMKII expression. Insulin infusion 24 h after an exercise bout for 4 h did not effect calpain-10 expression and this is consistent with the results in chapters 4 and 5 suggesting that prior exercise (and an increase in insulin sensitivity) does not affect the ability of insulin to regulate calpain-10 mRNA.

A recent study has examined more closely the effect of exhaustive cycling exercise on skeletal muscle calpain-3 and μ -calpain protein expression and autolysis in humans (Murphy et al., 2005). The results from this study showed that cycling exercise to exhaustion at 70% $\dot{V}O_2$ peak did not result in significant autolysis of calpain-3 or μ -calpain, which is in agreement to the data in the current study. The authors suggested that their exercise model did not produce an increase in intracellular calcium of sufficient duration to lead to increased calpain activity. In the present study, it is also possible that the intracellular

calcium concentrations were not elevated long enough to activate calpain, and this may have prevented any feedback induced changes in calpain expression. Given that the calcium induced activity of calpain-10, and to some extent calpain-3, has yet to be characterised and that calpain-10 activity cannot be measured directly or indirectly, it is currently unclear whether calpain-10 is activated by elevated calcium concentrations in cells. This would seem unlikely as calpain-10 lacks the typical calcium binding EF-hand motifs found in the ubiquitous calpains, as discussed in **chapter 1 (1.8)**. Nevertheless, it would be interesting to investigate the effects of an exercise bout of longer duration to that employed in this study (and therefore more sustained elevations in calcium concentrations) on calpain-10 and -3 expression.

GLUT4 and exercise

The finding that GLUT4 mRNA was not altered either immediately following exercise or 24 h after exercise may be surprising given that many studies have documented such a change in rodents. For example, Ren et al. (1994) showed that GLUT4 mRNA was upregulated two-fold 16 h after one prolonged swimming exercise session. In humans however, only a few studies have examined the effect of a single bout of exercise on GLUT4 mRNA expression. Kraniou et al. (2006 and 2000) have showed on two occasions that a single exercise bout increases GLUT4 mRNA in humans, although the mRNA data from the earlier publication has been called into question due to their

alleged inappropriate use of quantitative real-time PCR internal controls (Schjerling, 2001). There remains some controversy therefore in regards to the effect of a single bout of exercise on GLUT4 mRNA levels in humans. The data from the present study is in agreement with recent work which investigated the effect of a one-legged exercise bout on GLUT4 mRNA expression and found no effect (Vissing et al., 2005). In that study, subjects performed 3 h of one-legged cycling exercise at 50% $\dot{V}O_2$ peak and biopsy samples were taken immediately after exercise and during recovery from exercise. GLUT4 mRNA levels were found to be unchanged with the exercise bout or during 20 h of recovery when compared to resting controls (Vissing et al., 2005).

These results indicate that changes in the mRNA levels of GLUT4 in humans may not be associated with the increase in glucose uptake with exercise or the adaptive increase in insulin mediated glucose uptake during recovery from an exercise bout. In the short term therefore, increased GLUT4 translocation to the plasma membrane during exercise and during insulin infusion following exercise may play a more important role in the immediate and short term increases in glucose uptake into skeletal muscle. In the longer term and with repeated training, adaptive changes in the expression of GLUT4 in skeletal muscle may become more important and this has been demonstrated previously in humans (Helge et al., 2006; Kim et al., 2004; Dela et al., 1994).

Exercise regulation of pyruvate dehydrogenase kinase (PDK)

The pyruvate dehydrogenase complex plays a pivotal role in the entrance of pyruvate into the TCA cycle and thus is a major regulator of glucose oxidation in skeletal muscle. In this chapter, PDK4 mRNA was downregulated in the exercise leg immediately after the exercise bout when compared to the non-exercising leg. For the first time, this study also demonstrates the downregulation of PDK4 mRNA by insulin infusion. An additional isoform of PDK, PDK2, was also shown to be differentially expressed between the two legs with exercise; exercise led to an increase in PDK2 mRNA 24 h after the exercise bout and remained unchanged during the insulin clamp.

A number of studies have investigated the effects of a single bout of cycling exercise of various intensities and durations on PDK transcription and expression in human skeletal muscle (e.g., Pilegaard et al., 2000; Pilegaard and Neufer, 2004; Watt et al., 2004; Mourtzakis et al., 2006; Cluberton et al., 2005). In the first such study, PDK4 gene transcription was induced by both 75 min and 4 h of exhaustive exercise (Pilegaard et al., 2000). Both PDK4 transcription and mRNA expression was elevated at the end of each exercise bout and remained so throughout 4 h of recovery. However, both transcription and mRNA had returned to their pre-exercise levels 24 h following each exercise bout. Studying the time-course of PDK4 mRNA changes during moderate intensity exercise to exhaustion in more detail revealed that

PDK4 mRNA is not upregulated until 2 h into the exercise period (Pilegaard and Neufer, 2004). The current results show that 90 min of one-legged cycling exercise led to reduced PDK4 mRNA expression in the exercise leg versus the control leg. At the end of the exercise, whole body COX was substantially higher than FOX and therefore a reduced expression of PDK4 mRNA might be expected, as it would permit increased activation of the PDH complex and presumably increased COX. This idea was strengthened when, during the insulin infusion, PDK4 mRNA was reduced even further. The reason for the discrepancy between this and previous studies is not clear. In much of the previous work, the transcription rate of PDK4 was measured rather than mRNA content per se and this may account, at least in part, for the differences in the results and also in the interpretations. The duration and intensity of the exercise protocol is also likely to have a significant impact on substrate utilisation, PDK levels and PDH activity and this makes comparisons between studies difficult. Moreover, the pre exercise muscle glycogen content has been shown to blunt the effects of exercise on the metabolic gene expression and this may have been a contributing factor in the current study (Pilegaard et al., 2002).

PDK4 mRNA was reduced in both legs to the same extent 24 h after exercise in the current study and this is consistent with an increase in whole body glucose utilisation seen following exercise (Mikines et al.1988). These data suggest that a serum factor possibly linked to increased whole body insulin sensitivity may be responsible for the

reduction in PDK4 mRNA in both legs 24 h following exercise. The requirement for a serum factor in the improvements in insulin sensitivity with exercise has been demonstrated *in vitro* (Gao et al., 1994). It is currently not clear what this serum factor is but it is unlikely to be insulin, glucose or circulating FFA as these were no different 24 h after the exercise bout in the present study, but additional hormonal factors (which were not measured in this study) could also play a role. Other factors could also contribute to the reduction in PDK4 mRNA 24 h following exercise. For example, a change in pyruvate concentration and/or cellular energy status (NADH/NAD⁺, ATP/ADP) could affect PDK activity in the hours during recovery from exercise. A change in a number of transcription factors (FOXO1, PGC1 α , PPARs) have been previously documented during recovery from a single bout of endurance exercise (Mahoney et al., 2005) and it is possible that these factors could have contributed to the reduction in PDK4 mRNA expression. Interestingly, PGC1 α mRNA expression was increased 24 h following the exercise bout but only in the exercised leg. It is also possible that substrate availability and metabolic recovery (e.g. glycogen restoration) following exercise is regulating PDK4 mRNA expression in the present study. Some studies have noted an increase in PDK4 mRNA during recovery from exercise (Pilegaard et al., 2000; Pilegaard et al., 2005) and the difference between these studies and the present one is possibly a result of diet composition. It has been shown that altering the carbohydrate composition of the diet following exercise can have a

significant impact on PDK4 mRNA and transcription (Pilegaard et al., 2005).

The PDK4 mRNA data raise some interesting questions regarding the experimental protocol. Muscle biopsies were taken from both legs following the exercise period and the biopsy from the control leg, which was completely inactive during the exercise period, was used as a baseline. This was done to differentiate the effects of circulating hormonal factors (i.e. adrenaline) and exercise *per se* on gene expression. However, in so doing, both legs were exposed to the same circulating factors during the exercise bout and this could have had an impact on muscle metabolism and subsequent gene expression in the control leg. An important consideration in this is the effect of exercise on muscle glycogen levels in the control leg. Whilst this was not measured in the present study, previous studies using the one-legged cycling model have shown that muscle glycogen is unaffected in the control leg following exhaustive exercise in humans (Casey et al., 1995).

In contrast to PDK4, PDK2 mRNA was upregulated 24 h after the exercise bout and was not affected by insulin infusion in current study suggesting that the two isoforms are regulated by different mechanisms. One possible explanation for these results is that the relative amounts of each PDK isoform may determine the total PDK activity in certain physiological states. As PDK2 is more sensitive to

inhibition by pyruvate (Bowker-Kinley et al., 1998), a relative increase in PDK2 over PDK4 might be expected to increase the sensitivity of the PDK complex to inhibition by pyruvate and thus lead to an increase in PDH activity when carbohydrate is readily available.

Hexokinase II and PGC1 α

Finally, in this chapter it has been demonstrated that exercise increases the expression of HKII and PGC1 α . The finding that HKII is upregulated with exercise and during recovery from exercise is consistent with previous reports in humans (Koval et al., 1998; Pilegaard et al., 2005) and rats (O'Doherty et al., 1994). The maintenance of elevated mRNA levels of HKII in the exercised leg versus the control leg, combined with the alterations in PDK gene expression, may account for the relative increase in glucose uptake that occurs during recovery from exercise. Whilst the activity of HKII was not measured, it has been shown that exercise similarly increases the activity of HKII (Koval et al., 1998). In rats, low intensity swimming exercise increase the expression of PGC1 α (Terada et al., 2002) and similar findings have been demonstrated in humans (Pilegaard et al., 2003) and the results of the present study are consistent with these findings. It is thought that PGC1 α binds to and modulates the activity of specific transcription factors that are already bound to their target sequences. Elevated PGC1 α expression is thought to contribute to mitochondrial biogenesis via marked increases in the expression of a number of key metabolic

genes, including GLUT4 (Micheal et al., 2001). As mentioned previously, an increase in GLUT4 mRNA may not be necessary for the immediate or short term adaptations of skeletal muscle to a single bout of exercise. This may explain why PGC1 α mRNA was increased but GLUT4 mRNA was unchanged in the present. The present data also indicate that PGC1 α may control the expression of other metabolic genes that are important for the exercise mediated increase in insulin sensitivity but which were not measured in this study.

6.5 Conclusions

In conclusion, this study demonstrates that a single bout of exercise is associated with changes in the expression of genes important for glucose oxidation (PDK2, PDK4) and phosphorylation (HKII) but not glucose transport (GLUT4, calpain-10). This study also confirms the recent finding that the skeletal muscle specific calpain-3 is not affected by a concentric exercise protocol. These results provide novel insight into the molecular mechanisms behind not only the immediate effects of exercise on glucose uptake but also the pathways involved in the adaptive increase in insulin mediated glucose uptake seen during recovery from a single bout of exercise. Exercise is beneficial in the treatment of diabetes and an understanding of the molecular mechanisms responsible for the improvement in glucose uptake and insulin sensitivity during and after exercise may lead to novel strategies to combat diabetes that may be independent of insulin.

7 Calpain-10 single nucleotide polymorphisms and calpain-10 expression in type 2 diabetics

7.1 Introduction

Calpain-10 was one of the first genes to be identified as contributing to type 2 diabetes risk in Mexican Americans using a positional cloning approach (Horikawa et al., 2000). The risk could be most readily defined in terms of specific haplotypes of three polymorphisms: SNP-43, Indel-19 and SNP-63. The association of these SNPs and their haplotypes with type 2 diabetes has not been consistently replicated in other populations. Importantly for this thesis, this has particularly been the case in the UK (Evans et al., 2001) and other European populations (Fingerlin et al., 2002; Malecki et al., 2002; Orho-Melander et al., 2002). Evans et al. (2001) studied calpain-10 in white subjects of British/Irish ancestry using family based case control study designs. They examined the three SNPs mentioned above (SNP-43, Indel-19 and SNP-63) and an additional SNP (SNP-44) and did not find any association between risk for type 2 diabetes and SNP-43, Indel-19 and SNP-63 either individually or as part of the previously described risk haplotypes (Evans et al., 2001). They did observe increased transmission of the rare C risk allele at SNP-44 to affected offspring suggesting the possible involvement of alternative calpain-10 SNPs in UK populations (Evans et al., 2001). Lynn et al. (2002) investigated the

effects of variation in the calpain-10 gene on blood glucose and insulin levels in 285 nondiabetic British subjects following a 75 g oral glucose tolerance test. Their results indicated that those subjects with the at-risk genotype at SNP-43 had higher 2 h plasma glucose levels (Lynn et al., 2002). In addition, those with the at-risk haplotype also had increased fasting and 2 h plasma glucose and a reduced insulin secretory response (Lynn et al., 2002). The first part of this final chapter combines the data from all subjects used in this thesis and investigates the relationship between SNP-43, Indel-19 and SNP-63 and baseline measurements including blood glucose, serum insulin and plasma FFA levels and substrate oxidation rates. Where possible, data from insulin clamps was also combined to investigate the effect on glucose disposal.

Since the initial publication describing that variation within calpain-10 was associated with type 2 diabetes, there have not been any reports of investigations of the expression of calpain-10 in type 2 diabetics. This is surprising given that it is over 6 years since this initial finding. Given that reduced calpain-10 expression has been linked to insulin resistance in Pima Indians and is reduced with hyperinsulinaemia in this thesis, one would expect type 2 diabetics to have reduced levels of calpain-10. In the second part of this chapter, therefore, and as the final experiment in this thesis, the expression of skeletal muscle calpain-10 was measured for the first time in type 2 diabetics and compared to age, sex and activity matched control subjects.

7.2 Subjects and methods

7.2.1 Calpain-10 SNP study protocol

Every individual that participated in a study presented in this thesis was genotyped at SNP-43, Indel-19 and SNP-63 (**general methods, 2.5**) and these data were combined to allow for investigation into the effect of this SNP on blood metabolites, substrate oxidation rates and glucose disposal. It was not possible to examine the effect of the at-risk haplotype combination on these measurements due to the expected very low frequency of this haplotype. An extra group of subjects which were not used in any of the previous chapters described in this thesis were also genotyped and included in the subsequent analysis for this chapter (designated **chapter 7**). This group consisted of seven healthy men who underwent a standard 4 h hyperinsulineamic-euglyceamic clamp with measurements of fasting blood metabolites and basal and insulin mediated rates of COX and FOX. A summary of the combination of subjects and their characteristics is shown in table 7.1.

Table 7.1 Subject characteristics used for the analysis of calpain-10 SNPs

	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7	Total
n	10	11*	10	8	7	46
Age (yrs)	26 ± 1	26 ± 3	22 ± 1	24 ± 2	22 ± 2	24 ± 1
Body mass (kg)	81 ± 4	78 ± 3	78 ± 3	79 ± 4	79 ± 4	79 ± 2
BMI (kg.m ²)	26 ± 1	24 ± 1	24 ± 1	24 ± 1	25 ± 1	24 ± 0.4

Subjects from each chapter were combined with the additional group of subjects (designated chapter 7) for the analysis of calpain-10 SNPs. The characteristics of the combined group that was used in this chapter is shown in the last column (total). *An extra subject who did not complete the high fat study was also included in the present study.

7.2.1.1 Calpain-10 SNP determination

Each individuals genotype at SNP-43, Indel-19 and SNP-63 was determined as described in the **general methods (2.5)** chapter.

7.2.1.2 Blood metabolites

Blood glucose, serum insulin and plasma FFA concentrations were determined for each individual in each chapter as described in the **general methods (2.2.2)**. Plasma FFA were not determined in the additional group. Fasting data for blood metabolites were combined and analysed in terms of SNP genotype.

7.2.1.3 Substrate oxidation

Estimates of basal and insulin mediated substrate oxidation rates were obtained in **chapters 4** and **5** and in the additional group of subjects (**chapter 7**) using exactly the same equipment and procedure as described in the **general methods (2.2.4)**. In **chapter 6**, only fasting data was included in the analysis. Data were expressed as $\mu\text{mol/kg/min}$ in each case and then combined and analysed in terms of the SNP genotype.

7.2.1.4 Glucose disposal

Data from baseline insulin clamps, that is prior to any intervention, in **chapters 4** and **5** and from the additional group of subjects (**chapter 7**) were combined and analysed in terms of the SNP genotype. A 6 h clamp was performed in **chapter 5**, and to allow for combination with the additional data, only the 4 h clamp data were used in the subsequent analysis.

7.2.2 Type 2 diabetes study protocol

7.2.2.1 Subjects

This study was performed in collaboration with Dr L.J.C. van Loon and colleagues at Maastricht University, The Netherlands. All physiological procedures described in the following section were performed by Dr L.J.C van Loon and colleagues. Briefly, a total of 10 sedentary, overweight type 2 diabetes patients (60.0 ± 2.0 yr), 10 sedentary, weight-matched, normoglycaemic controls (60.0 ± 2.0 yr) and 10 age-matched endurance trained cyclists (57.0 ± 1.0 yr) were selected to participate in this study (Table 7.3). All type 2 diabetes patients were using oral blood glucose lowering medication (metformin with or without sulfonylurea derivatives). Type 2 diabetic status was verified with an

oral glucose tolerance test (OGTT) according to World Health Organisation (WHO) criteria.

7.2.2.2 Subject screening

All subjects performed an oral glucose tolerance test (OGTT). After an overnight fast, subjects arrived at the laboratory at approximately 8 am and a catheter (Baxter BV, Utrecht, the Netherlands) was inserted into an antecubital vein and a resting blood sample was drawn. Following this, 75 g of glucose dissolved in 250 ml water was ingested and blood samples were taken at 2 h post glucose ingestion. Blood glucose concentrations were measured to obtain an index of glucose tolerance according to the American Diabetes Association (ADA) guidelines of 2003. Maximal power output (W_{max}) and maximal oxygen uptake capacity ($\dot{V}O_2 \text{ max}$) were determined on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) during an incremental exhaustive exercise test 2 weeks before obtaining a muscle biopsy sample. Oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were measured continuously (Oxycon β , Mijnhart, Breda, The Netherlands). Residual lung volume was measured by the helium-dilution technique using a spirometer (Volugraph 2000, Mijnhart, Bunnik, The Netherlands). Body composition was assessed using the hydrostatic weighing method in the morning after an overnight fast and body weight was measured with a digital balance with an accuracy of

0.001 kg (E1200, August Sauter GmbH, Albstadt, Germany). Body fat percentage was calculated using Siri's equation and fat free mass (FFM) was calculated by subtracting fat mass (FM) from total body weight.

7.2.2.3 Muscle sampling and analysis

A fasting muscle biopsy was obtained from all patients as described in the **general methods (2.2.7)**. Calpain-10 mRNA and protein levels were determined also as previously described in the **general methods (2.2.7)**.

7.2.2.4 Calpain-10 immunohistochemistry

This procedure was performed by Rene Koopman at Maastricht University, The Netherlands. Briefly, muscle samples were dissected and frozen in liquid nitrogen. Approximately 15 mg of muscle were frozen in nitrogen-cooled isopentane and embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands). Multiple serial sections (5 µm) were thaw mounted on uncoated, pre-cleaned glass slides, fixed in formaldehyde and incubated overnight with calpain-10 primary antibody (N7) at a concentration of 1:100. After washing with TBS-T, the slides were incubated with a secondary IgG antibody labelled with FITC for 30 min followed by 3 wash steps with TBS-T.

Slides were examined using a Nikon E800 fluorescence microscope (Uvikon, Bunnik, The Netherlands) coupled to a Basler A113 C progressive scan colour charge-coupled device (CCD) camera. The antibody 4'-6-Diamidino-2-phenylindole (DAPI) was used as control for nuclear staining.

7.3 Results

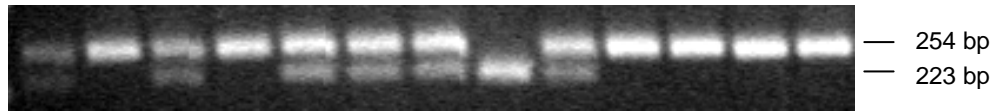
7.3.1 Calpain-10 SNP analysis

As shown in Fig 7.1, genotyping each individual at SNP-43, Indel-19 and SNP-63 was carried out successfully using the RFLP method. The frequency of each allele is given in Table 7.2 and the numbers of each particular genotype are given in Table 7.3. In total, 46 individuals were included in this study (Table 7.1) and all could be genotyped except for one subject at SNP-43 and two individuals for both Indel-19 and SNP-63.

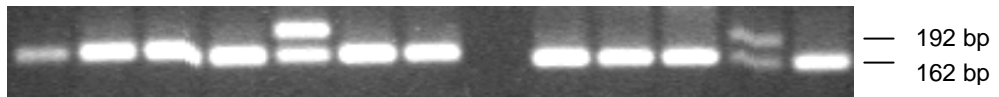
7.3.1.1 SNP and subject characteristics

There was no significant effect of the SNP-43, Indel-19 or SNP-63 genotype individually on blood metabolites, rates of carbohydrate and fat oxidation or insulin stimulated glucose disposal, as summarised in Table 7.4. There was not sufficient numbers of subjects to analyse the effect of the at-risk haplotype combination; only a single subject had the at-risk genotype in this study.

A



B



C

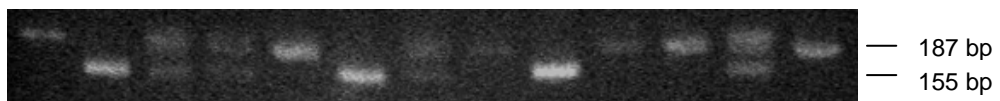


Figure 7.1 Representative agarose gels of digested PCR products for SNP-43 (A), SNP-63 (B) and Indel-19 (C).

PCR products were digested with the appropriate restriction enzyme and run on 3% (w/v) agarose gels. For SNP-43, the G and A alleles are seen as bands of 254 bp and 223 and 31 bp, respectively. Allele 1 (C) of SNP-63 is seen as a product of 162 bp and allele 2 (T) is observed as a band of 192 bp. Indel-19 is seen as bands of 187 bp (three repeats) and 155 bp (two repeats).

Table 7.2 Allele frequencies for SNP-43, Indel-19 and SNP-63

	SNP-43	Indel-19	SNP-63
Allele 1	0.77	0.44	0.93
Allele 2	0.23	0.56	0.07

SNP-43: allele 1 (G), allele 2 (A). Indel-19: allele 1 (two repeats), allele 2 (three repeats). SNP-63: allele 1 (C), allele 2 (T)

Table 7.3 Number of each genotype at SNP-43, Indel-19 and SNP-63

	SNP-43	Indel-19	SNP-63
1/1	26	10	39
1/2	17	19	4
2/2	2	15	1
Total	45	44	44

A total of 46 subjects were included in this study. 45 could be genotyped at SNP-43 and 44 at Indel-19 and SNP-63. Genotype 1/1 and 2/2 refers to those homozygous for allele 1 and allele 2, respectively, and 1/2 refers to those heterozygous for these alleles. A description of the alleles is given in Table 7.2.

Table 7.4 The effect of SNP-43, Indel-19 and SNP-63 on blood metabolites, substrate oxidation and glucose disposal.

	SNP-43		Indel-19			SNP-63		
	1/1	1/2 + 2/2	1/1	2/2	1/2	1/1	2/2	1/2
Glucose (mmol/L)	4.47 ± 0.05 (26)	4.39 ± 0.07 (19)	4.39 ± 0.09 (10)	4.49 ± 0.10 (15)	4.46 ± 0.05 (19)	4.48 ± 0.05 (39)	4.16 (1)	4.33 ± 0.15 (4)
Insulin (mU/L)	6.45 ± 0.49 (26)	6.15 ± 0.50 (19)	5.36 ± 0.61 (10)	6.97 ± 0.68 (15)	6.92 ± 0.62 (19)	6.72 ± 0.42 (39)	7.25 (1)	4.57 ± 0.65 (4)
FFA (mmol/L)	0.39 ± 0.05 (18)	0.36 ± 0.04 (16)	0.42 ± 0.09 (7)	0.41 ± 0.10 (9)	0.39 ± 0.05 (11)	0.39 ± 0.04 (31)	0.19 (1)	0.34 ± 0.07 (3)
COX _{basal}	10.6 ± 1.4 (23)	9.63 ± 0.9 (14)	9.3 ± 1.6 (8)	9.13 ± 1.3 (12)	10.9 ± 1.3 (17)	10.3 ± 0.8 (35)	ND	5.0 ± 0.8 (2)
COX _{insulin} (µmol/kg/min)	22.9 ± 2.8 (20)	20.4 ± 1.5 (8)	20.7 ± 3.2 (7)	19.2 ± 2.7 (9)	22.7 ± 1.7 (13)	21.2 ± 1.4 (26)		20.9 ± 1.7 (2)
FOX _{basal}	5.8 ± 0.6 (23)	6.0 ± 0.4 (14)	6.2 ± 0.4 (8)	6.1 ± 0.4 (12)	5.7 ± 0.6 (17)	5.9 ± 0.3 (35)	ND	7.7 ± 0.4 (2)
FOX _{insulin} (µmol/kg/min)	1.9 ± 0.8 (20)	2.8 ± 0.5 (8)	3.2 ± 0.7 (7)	2.9 ± 0.8 (9)	2.0 ± 0.6 (13)	2.6 ± 0.5 (26)		2.5 ± 0.1 (2)
CHO disposal (µmol/min/kg)	59.5 ± 3.3 (18)	60.4 ± 4.5 (9)	60.4 ± 4.9 (7)	57.2 ± 3.1 (9)	60.3 ± 4.2 (11)	58.3 ± 2.3 (28)	ND	62.3 ± 2.3 (2)

Blood glucose, serum insulin and plasma FFA, rates of carbohydrate (COX) and fat (FOX) oxidation and insulin mediated glucose disposal were analysed in terms of each subjects' genotype at SNP-43, Indel-19 and SNP-63 as described. The number of subjects in each category is shown in brackets. Where there were no subjects in a category, results were not determined (ND). Refer to Table 7.3 for genotype information. Data are mean ± SEM.

7.3.2 Type 2 diabetes and calpain-10 expression

In this chapter, the expression of calpain-10 mRNA and protein in the skeletal muscle of type 2 diabetics and was compared to the expression in healthy age matched and endurance trained control subjects (Table 7.3). Calpain-10 mRNA was no different between the three groups (CON 0.89 ± 0.05 vs. DIABETICS 0.82 ± 0.05 vs. TRAINED 0.90 ± 0.04) (Fig 7.2A). For calpain-10 protein, immunoreactive bands were seen at 75, 60, 48 and 35 kDa as in **chapter 5 (5.3.3)** and these were quantified in each group. Whilst there was no significant difference in the expression of any of the bands individually between each group, interesting trends in the expression of the 60 and 48 kDa bands were observed on the Western blot in some subjects and this is shown in Fig 7.2B. This led to the quantification of all of the observed calpain-10 bands for the analysis of total calpain-10 protein. When the expression of the bands was combined there was a strong trend towards reduced calpain-10 protein in the diabetic group compared to the trained subjects (DIABETICS 0.55 ± 0.05 vs. TRAINED 0.67 ± 0.10 , $P=0.06$) (Fig 7.2B). There was no significant difference in total calpain-10 protein between the sedentary control and diabetic group, or between the control and trained group (Fig 7.2B). Immunohistochemistry of control muscle showed that calpain-10 was highly localised to the nucleus, cell membrane and vasculature in skeletal muscle (Fig 7.4).

Table 7.5 Subjects' characteristics in the type 2 diabetic trial

	Type 2 Diabetes	Sedentary Controls	Trained Controls
	n = 10	n = 10	n = 10
Age (yrs)	58.9 ± 2.5	60.0 ± 1.9	57.4 ± 0.8
Height (m)	1.79 ± 0.02	1.76 ± 0.01	1.75 ± 0.01
Body mass (kg)	93.0 ± 4.4	86.9 ± 1,9	77.7 ± 1.8 #
BMI (kg.m ²)	28.9 ± 1.2	27.5 ± 0.5	25.5 ± 0.7 #
Body fat (%)	30.4 ± 1.8	28.9 ± 1.4	17.2 ± 11.2 *#
Fat free mass (kg)	64.5 ± 2.4	61.7 ± 1.6	64.2 ± 11.2
Basal plasma glucose (mM)	9.0 ± 0.4	5.5 ± 0.2 #	5.7 ± 0.1 #
Plasma glucose _{120min} (mM)	16.81 ± 1.0	5.34 ± 0.49 #	5.28 ± 0.4 #
Basal plasma insulin (mU/L)	8.70 ± 1.01	7.86 ± 1.58	5.13 ± 0.56
Plasma insulin _{120min}	47.24 ± 9.61	48.40 ± 8.04	29.40 ± 6.34
HbA1c (%)	7.30 ± 0.3	5.83 ± 0.2 #	5.78 ± 0.1 #
VO ₂ max (L/min)	2.9 ± 0.2	3.2 ± 0.2	3.8 ± 0.1 #
Wmax (W)	205 ± 16	206 ± 18	300 ± 9 *#
Maximal heartrate (bpm)	161 ± 4	164 ± 7	172 ± 3
Diagnosed with diabetes (yrs)	7 ± 1	-	-

Plasma glucose/insulin_{120min} represents plasma glucose/insulin concentrations at t = 120 min during the OGTT. *P<0.05 vs. sedentary control group, #P<0.05 vs. type 2 diabetes group.

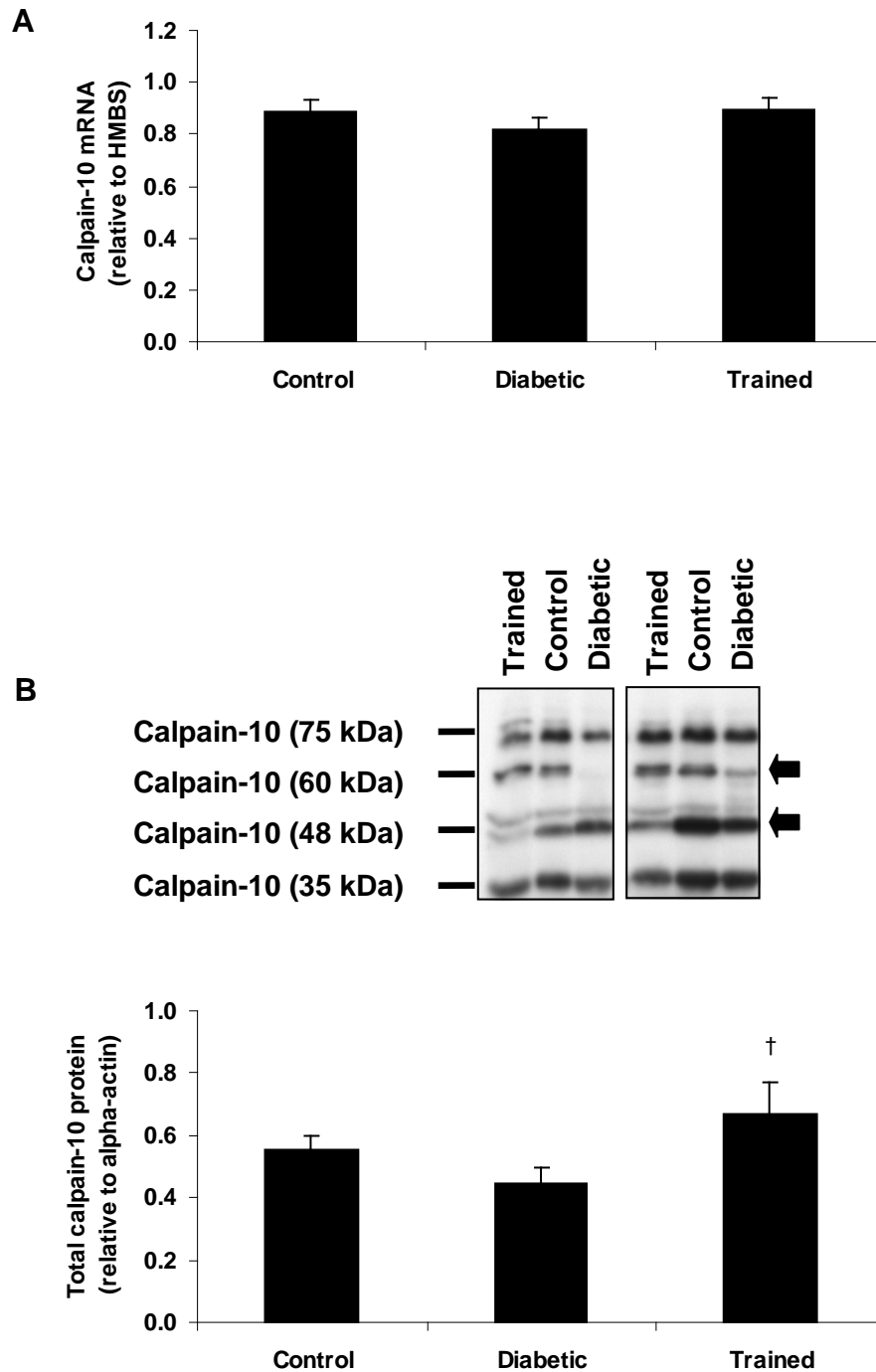


Figure 7.2 Calpain-10 mRNA (A) and protein expression (B) in type 2 diabetics and healthy control and trained subjects.

A representative Western blot shows the detection of multiple immunoreactive bands for calpain-10 (B), some of which were differentially expressed (arrows). All of these bands were quantified and expressed as total calpain-10 protein (B). [†]P<0.1 vs. diabetic group.

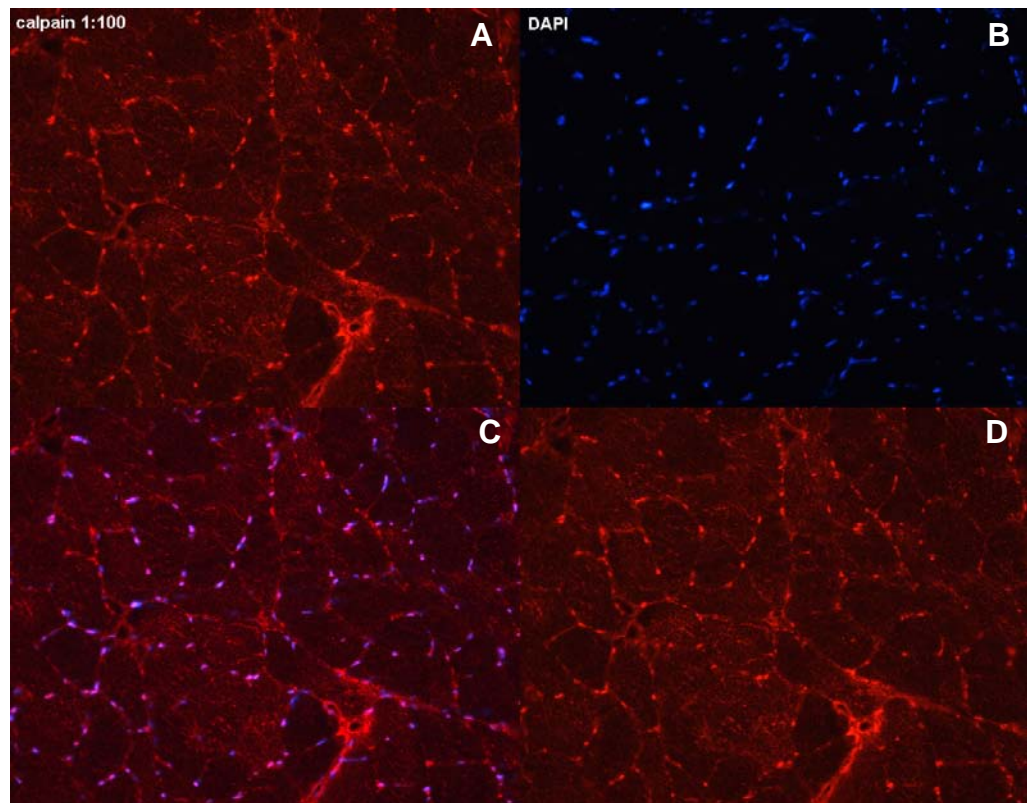


Figure 7.3 Immunohistochemistry of calpain-10 in human skeletal muscle.

Panels A and D show calpain-10 staining in red using the N7 antibody (1:100) and panel B shows nuclear staining in blue. Panel C merges both calpain-10 (A) and DAPI (B) signals and demonstrates calpain-10 localisation at the nucleus and plasma membrane.

7.4 Discussion

This study examined the effect of the common calpain-10 polymorphisms SNP-43, Indel-19 and SNP-63 on blood metabolites, insulin mediated glucose disposal and basal and insulin mediated COX and FOX in healthy subjects. The expression of calpain-10 in the skeletal muscle of type 2 diabetics and age matched healthy and trained control subjects and the immunolocalisation of calpain-10 was also investigated. The main findings from this final chapter are that the none of the SNPs, including the at-risk SNP-43 G/G genotype, were associated with fasting blood glucose, serum insulin or plasma FFA levels, insulin stimulated glucose disposal or rates of COX and FOX. In the second part of this chapter, it was shown for the first time that total calpain-10 protein levels were lower in type 2 diabetic patients when compared to age matched endurance trained, but not sedentary, control subjects.

A number of studies have previously shown that SNP-43 is associated with a number of diabetes related traits, such as elevated blood glucose (Lynn et al., 2002), serum cholesterol (Daimon et al., 2002), plasma FFA (Orho-Merlander et al., 2002; Carlsson et al., 2004) serum insulin concentrations (Orho-Merlander et al., 2002) and recently abdominal obesity (Pihlajamaki et al., 2006). The same polymorphism has been linked to reduced calpain-10 mRNA, lower rates of postabsorptive and insulin stimulated glucose turnover (Baier et al., 2001; Tripathy et al.,

2004) and a reduced ability to oxidise carbohydrate (Baier et al., 2001). On the other hand, other studies have failed to demonstrate such an association and this is particularly the case in the UK and other European populations. For example, Evans et al. (2001) did not show an association between SNP-43 and type diabetes in the UK population, but were able to show an association with the C allele at an additional SNP-44. The present study would appear to strengthen these findings for SNP-43 as no association between this SNP and type 2 diabetes related traits was seen, but the role of SNP-44 in this study remains unknown.

It is not at all clear how SNP-43 would affect any of the physiological parameters outlined in this chapter. Elucidating the role of this polymorphism has been confounded further by the fact that SNP-43 is located within intron-3 (a region of DNA that is spliced out after transcription) of calpain-10. Horikawa and colleagues considered the possibility that there was a gene embedded in intron-3 and whether intron-3 influenced calpain-10 transcription as a result (Horikawa et al., 2000). However, they were not able to detect a signal on northern blots probed using intron-3 suggesting that intron-3 is not part of another gene. In separate experiments the G allele displayed much lower binding to a factor present in nuclear extracts from HepG2 and human pancreatic cells when compared to the A allele. Cloning of fragments of intron-3 containing SNP-43 (and also SNP-44) upstream of an SV40 promoter-luciferase gene transcriptional unit revealed that intron-3 and

the SNP-43 (and SNP-44) were able to differentially regulate the expression of the SV40 reporter gene (Horikawa et al., 2000). Others have since shown that calpain-10 mRNA is reduced considerably in human skeletal muscle of subjects homozygous for the G allele at SNP-43 (Baier et al., 2000; Carlsson et al., 2005). It was not possible in the present study to examine the effect of SNP-43 on calpain-10 mRNA and protein expression due to the difficulties in comparing across Taqman real-time PCR plates and Western blots. When looking at the effect of SNP-43 on calpain-10 mRNA in the individual chapters, however, there was no differences between the G/G and G/A and A/A group in any chapter. The link between calpain-10 SNP-43, calpain-10 expression and phenotypes relating to type 2 diabetes therefore remains unknown.

In general, a low number of subjects in this thesis made it difficult to investigate the role of all of the calpain-10 SNPs in detail, although this is the first study to examine the role of calpain-10 SNPs on substrate oxidation and glucose disposal in healthy UK subjects. Despite the fact that all subjects were combined in the present study, it is important to consider that the present study is likely to be underpowered as it was calculated that to detect a significant effect of the SNP-43 genotype on insulin mediated glucose disposal, a total of 20 subjects was required in each group. To detect a difference in insulin mediated COX, one would require in excess of 40 subjects per genotype group. A lack of statistical power was particularly evident for the at-risk polymorphisms

which were much less abundant, for example the rare alleles at SNP-63 and also SNP-44, which was not genotyped in this thesis due to the very rare occurrence of the risk C allele. This also made it difficult to examine the role of the risk haplotype combination as only a single subject was positive for this haplotype. It is tempting to speculate, however, that the subjects' response (i.e. calpain-10 expression) to the physiological interventions in this thesis may have been governed by their genotype at particular loci, so-called environment-gene interactions. Interestingly, in the high fat study of this thesis (**chapter 4**), it was observed that subjects who were homozygous for the at-risk G allele at SNP-43 were more profoundly affected by the high fat diet intervention and had lower basal glucose disposal and reduced COX compared to those who had at least one copy of the A allele. These results did not reach statistical significance, most likely because of the low number of subjects in each group. Therefore, future investigations *in vitro* and *in vivo* with more subjects whose genotype would be predetermined would be beneficial for studies into calpain-10, type 2 diabetes and calpain-10 nutrient-gene interactions.

This chapter includes the first measurement of calpain-10 mRNA and protein in the skeletal muscle of type 2 diabetics. There was no significant difference in calpain-10 mRNA between either the control group or the diabetic group. This result was perhaps surprising in the context of the currently available literature and given that this gene has been closely linked to type 2 diabetes, but perhaps not surprising in the

context of the findings of this thesis. Whilst it has been shown in this thesis that hyperinsulinaemia can downregulate calpain-10 expression, the type 2 diabetic patients included in this chapter were not hyperinsulinaemic and this may partly explain why calpain-10 mRNA levels were not different in the diabetics when compared to the control subjects. Interestingly, however, there was a strong trend towards reduced total calpain-10 protein in the skeletal muscle of the diabetic patients, when compared to the endurance trained control subjects. Although there was no significant difference in the expression of any of the immunoreactive bands individually, most of the difference in the total calpain-10 protein was accounted for by reductions in the expression of smaller bands. This may indicate a potential role for calpain-10 isoforms in the development of type 2 diabetes. It has been stressed previously in this thesis that consideration of calpain-10 isoforms is important but results from isoform expression studies (including the present study) must be interpreted with caution given that none of the bands have been sequenced and no information is available regarding the regulation and protein expression of calpain-10 isoforms. Nevertheless, this is an interesting observation given that endurance trained athletes have elevated expression of genes that are important for glucose transport (e.g. GLUT4) (Richter et al., 1998; Kim et al., 2004; Short et al., 2003; Langfort et al., 2003; Dela et al., 1993; Gulve and Spina, 1995) and oxidation (e.g. PDH) (Pilegaard et al., 2000; LeBlanc et al., 2004). Endurance training is also well known to increase insulin mediated glucose uptake (Dela et al., 1992) and it is

possible that the elevated calpain-10 and GLUT4 protein levels may, in part, govern this muscle adaptation to training. The endurance trained subjects used in the present study may also have had differences in muscle fibre type composition when compared to the diabetic subjects and this may partly explain the difference in calpain-10 expression. Interestingly, the expression of calpain-3 has been shown to be increased in type II fast fibres (Jones et al., 1999). In support of this idea, immunohistochemistry of calpain-10 in the present study suggested that calpain-10 expression may be regulated by muscle fibre type as some muscle fibres clearly stained more intensely than others. Whilst these are interesting preliminary observations, further studies are needed to examine more closely the effect of fibre type on calpain-10 expression.

Another interesting and novel observation in the present study is that calpain-10 protein appears to be localised to the nucleus and sarcolemma in human skeletal muscle. This finding is in close agreement with the results of Ma et al. (2001) who demonstrated that calpain-10 was localised to the sarcolemma in skeletal muscle fibres from young mice and in the nucleus of lens epithelial cells (Ma et al., 2001). These results are also consistent with computer-aided PSORT analysis for nuclear localisation signals in domain III of calpain-10 (Ma et al., 2001) and findings from Gafni et al. (2004) who demonstrated the nuclear localisation of calpain-10 isoforms in human kidney 293T cells *in vitro*. Together these findings are in contrast to a more recent study

suggesting that calpain-10 is located in rat and rabbit kidney mitochondria (Arrington et al., 2006). Attempts to detect calpain-10 in human skeletal muscle mitochondria during the course of this thesis have repeatedly failed indicating that calpain-10 is not located in the mitochondria in humans. An unexpected finding in the present study was that calpain-10 appeared to be highly localised to the skeletal muscle microvasculature. Whilst also a preliminary observation, this finding could have important implications for the future study of calpain-10 in muscle. The proposed association between skeletal muscle insulin resistance and calpain-10 could be a result of the role of calpain-10 in insulin resistance at the level of the vasculature. Insulin can exert control over capillary surface area via activation of nitric oxide synthase (NOS) and an increase in nitric oxide (NO) release and can therefore increase total blood flow and blood volume in skeletal muscle. Microvascular dysfunction has been linked to insulin resistance and it has been suggested that it may form the basis of the metabolic syndrome (Serne et al., 2006). This is based on the fact that the ability of insulin to dilate skeletal muscle vasculature is impaired in insulin resistant states (Serne et al., 2006). Any disruption to the correct delivery of insulin and glucose to muscle will have implications for insulin mediated glucose uptake; thus the vasodilatory and metabolic actions of insulin may be coupled (Serne et al., 2006). Interestingly, the vascular actions of insulin that stimulate NO production are PI3K dependent and are very similar to metabolic insulin signalling pathways providing a potential mechanism for this coupling (Serne et al., 2006).

A role for calpain-10 in these pathways could be one way in which it exerts an influence on insulin sensitivity. Clearly more work is needed to define the subcellular localisation of calpain-10 in skeletal muscle and other insulin sensitive tissues that are important for insulin resistance and type 2 diabetes.

7.5 Conclusions

This chapter demonstrates that in healthy male subjects calpain-10 polymorphisms are not associated with alterations in fasting blood metabolite concentrations, basal and insulin mediated substrate oxidation rates or insulin stimulated glucose disposal. It is also shown that type 2 diabetic subjects do not have reduced levels of calpain-10 mRNA or protein when compared to age matched controls. Endurance trained athletes, however, tended to have elevated levels of calpain-10 protein when compared to the type 2 diabetic group indicating that calpain-10 may be important for the skeletal muscle adaptations that occur with endurance training. This is the first study to measure the expression of calpain-10 in type 2 diabetics and athletes and more work is needed to more fully understand the regulation of calpain-10 expression in these populations.

8 Final discussion

The major aim of this thesis was to test the hypothesis that skeletal muscle calpain-10 and -3 gene and protein expression would be affected in states of altered insulin sensitivity in healthy humans given their proposed role in skeletal muscle metabolism, type 2 diabetes and insulin resistance. In order to test this hypothesis, a number of human *in vivo* experiments were performed, all of which were designed to either reduce or increase whole body insulin sensitivity and assess the mRNA and protein expression of calpain-10 and -3 in skeletal muscle. This thesis also set out to investigate the role of genetic variation in the calpain-10 gene on carbohydrate utilisation in healthy humans. In the final chapter the expression of calpain-10 in type 2 diabetic patients was compared to control and endurance trained subjects.

In summary, the major finding of this thesis is that calpain-10 and -3 expression is not altered when insulin sensitivity is negatively or positively regulated. Short-term fasting (**Chapter 3**) and Intralipid infusion (**Chapter 5**) led to insulin resistance but had no effect on calpain-10 or -3 mRNA and protein expression. This was despite the fact that short-term hyperinsulinaemia (achieved by insulin clamps) resulted in reduced skeletal muscle calpain-10 mRNA expression (**Chapter 5**). This effect appeared to be independent of the reduction in insulin sensitivity and glucose uptake during the insulin clamp as lipid infusion for 6 h markedly reduced insulin mediated glucose uptake but

did not modify the response of calpain-10 mRNA to insulin. Moreover, an Increase in insulin sensitivity with a single bout of moderate intensity exercise led to increased skeletal muscle glucose uptake but had no effect on calpain-10 or -3 mRNA expression (**Chapter 6**). This thesis also demonstrates that variation in the calpain-10 gene, previously linked to type 2 diabetes in some populations, was not associated with insulin mediated carbohydrate disposal or oxidation in healthy British subjects (**Chapter 7**). Neither was any difference found in the mRNA expression of calpain-10 in type 2 diabetic patients when compared to sedentary and endurance trained control subjects (**Chapter 7**). Interestingly, the protein expression of calpain 10 was found to be lower in the diabetic patients when compared to the trained, but not the sedentary, controls (**Chapter 7**).

At the commencement of this thesis project and in the years following the publication of the initial association (Horikawa et al., 2000), there was considerable interest in calpain-10 as it was the first gene to be associated with type 2 diabetes using a positional cloning approach. A genome wide linkage study with affected sib-pairs for type diabetes genes in a Mexican American population localised a type 2 diabetes susceptibility gene (NIDDM1) to chromosome 2 (Hanis et al., 1996; Cox et al., 1999). Single nucleotide polymorphisms (SNPs) were identified in this region and initially SNPs were surveyed in eight patients of families with evidence of linkage at NIDDM1 and in two patients of families without evidence for linkage. Those SNPs with minor allele

frequencies of more than 10% or those showing a unique pattern were genotyped in a patient group of 110 Mexican Americans with type 2 diabetes and a control group of 112 subjects to compare allele and haplotype frequencies between the two groups. No single SNP was significantly associated with type 2 diabetes but when three SNPs were combined (SNP-43, Indel-19 and SNP-63) a significant association with type 2 diabetes was found. This combination was also associated with diabetes in Finnish and German populations (Horikawa et al., 2000).

Whilst most studies then focused on confirming this association in a variety of populations using molecular genetic approaches, on close examination of the literature, a number of potential obstacles became apparent. Firstly, the “at-risk” SNPs were all located in intronic regions of the calpain-10 gene (Horikawa et al., 2000). In other words, the SNPs that were associated with type 2 diabetes were located in genetic regions that did not code for, or alter, the calpain-10 protein. It was shown in the original association study that intron 3, which contained SNP-43, may contribute to calpain-10 splicing generating calpain-10f (Horikawa et al., 2000). However, this is probably a rare event and in any case calpain-10f is one of the smallest calpain-10 isoforms and lacks a complete domain II, which is the critical catalytic domain in the ubiquitous calpains. Whilst it was also shown that the SNP-43 region could affect calpain-10 transcription *in vitro* (Horikawa et al., 2000), a direct relationship between SNP-43 (and the other SNPs for that matter) and calpain-10 transcription has not been demonstrated *in vivo*; only

SNP-43 has been associated with reduced calpain-10 mRNA levels since the initial association study (Baier et al., 2000; Carlsson et al., 2005). No study up to the end point of this thesis had even attempted to measure calpain-10 protein in human skeletal muscle (a major site of insulin resistance) and therefore it has not been demonstrated that any of the risk SNPs can affect calpain-10 protein levels.

Another drawback with the initial association study findings was that there was no obvious mechanism whereby calpain-10 could affect metabolism in such a way as to have a major impact on diabetes risk. Calpain-10 was a completely unknown gene and its function was not immediately apparent. As outlined in chapter 1, the calpains had been linked to a variety of functions, including skeletal muscle growth and differentiation, but not glucose utilisation or any other role that would have an obvious impact on insulin resistance and type 2 diabetes. What was also unknown was whether calpain-10 was actually a protease and/or whether it was activated by calcium. Calpain-10 lacks typical calcium binding domains of the ubiquitous calpains but the active site triad is conserved in calpain-10.

Only a handful of functional studies were performed following the initial association and, to date, these studies are extremely limited in nature. Most have been performed using cell cultures *in vitro* and have used general non-specific calpain inhibitors to examine the role of calpain-10 primarily in glucose uptake in the adipocyte (e.g. Paul et al., 2003) and

in insulin release from the pancreas (e.g. Marshall et al., 2005). Of the few studies that focused on glucose uptake suggested that calpain-10 may have some role in GLUT4 mediated pathways of glucose uptake *in vitro*. More convincing evidence currently exists for a role for calpain-10 in insulin release in the pancreas (Marshall et al., 2005). Nevertheless, evidence continued to mount from the association studies with many reporting positive links between calpain-10 genetic variants and type 2 diabetes. Meta-analyses of many of the population studies appeared to confirm that calpain-10 was associated with type 2 diabetes (Weedon et al., 2003; Song et al., 2004; Tsuchiya et al., 2006). This was even the case in the UK population where studies had indicated that calpain-10 variants affect blood glucose levels (Evans et al., 2001).

The important point is that at the time of this thesis, there was good evidence to suggest that calpain-10 may play a role in the development of insulin resistance and type 2 diabetes. At the very least, this evidence existed at the level of association studies with some additional, albeit indirect, evidence coming from the limited functional *in vitro* data. What was clearly lacking was studies performed *in vivo* in humans demonstrating that states of insulin resistance were functionally linked to changes in calpain-10 mRNA and protein expression in skeletal muscle, which is the major site for insulin mediated glucose disposal. The hypothesis of this thesis was therefore relatively simple – that changes in whole body and skeletal muscle insulin sensitivity in

otherwise healthy humans would alter the expression of calpain-10 mRNA and protein in skeletal muscle.

In the first two experimental chapters of this thesis, it can be argued that part of this aim was not achieved, i.e. that insulin resistance at the level of skeletal muscle was not achieved or that the magnitude in the reduction in skeletal muscle insulin sensitivity was relatively small and short lived. In the first experimental chapter, short-term fasting did lead to whole body insulin resistance but the contribution of skeletal muscle to this insulin resistance was uncertain as an insulin tolerance test was used to estimate insulin sensitivity. Previous studies using an almost identical fasting protocol in humans did demonstrate skeletal muscle insulin resistance (Mansell and Macdonald et al., 1990) and, combined with the reduction in skeletal muscle GLUT4 content, it seems likely that skeletal muscle insulin resistance was achieved, although the extent of this insulin resistance is unknown. In the following chapter, however, a one-week high-fat diet led to a slight increase in insulin mediated glucose disposal and only mild reductions in carbohydrate and fat oxidation. It is probable that the lack of insulin resistance seen in this chapter was due to the relatively short duration of the high-fat feeding protocol. In these two studies, therefore, it was perhaps not surprising that calpain-10 mRNA and protein expression was not affected, particularly in chapter 4 where no reduction in insulin mediated glucose disposal was seen at all. The first two interventions in this thesis were probably associated with early adaptations in the expression and

activity of enzymes important in the regulation of carbohydrate metabolism and not necessarily reductions in insulin signalling pathways. This may be an additional explanation for the lack of effect of these interventions on skeletal muscle calpain-10 expression as there is currently no evidence to suggest that calpain-10 may play a role in these early physiological adaptations.

However, in chapter 5 there was a more pronounced reduction in insulin mediated glucose disposal with Intralipid infusion. Previous studies have also demonstrated that this intervention is associated with significant alterations of the insulin signalling pathway (Belfort et al., 2005). In this study, infusion of Intralipid alone had no effect on calpain-10 expression but infusion of insulin for 6 h with or without the simultaneous infusion of Intralipid significantly reduced calpain-10 mRNA expression. This finding was unexpected in the context of chapter 4, in which insulin was infused for only 4 h, but is also consistent with these clamps as the reduction in calpain-10 mRNA was only significant at the end of the clamp in chapter 5 (6 h) and not half way through the clamp (3 h). The level of the hyperinsulinaemia seen in chapter 5 may also have contributed to the significant reduction in calpain-10 expression as in chapter 5, insulin concentrations during the clamp were higher than in previous chapters (~90 mU/L in chapter 5 vs. 70 mU/L in chapter 4). Whilst the reason for this difference is unknown, but are most likely due to subject variability, these data suggest that calpain-10 is regulated by hyperinsulinaemia per se and may not be

regulated by states of insulin resistance or changes in substrate utilisation at rest as Intralipid infusion also led to marked reductions in carbohydrate oxidation.

It is therefore tempting to speculate that the regulation of calpain-10 by hyperinsulinaemia may explain, in part, the finding of reduced calpain-10 in insulin resistant states as many of these (i.e. obesity, type 2 diabetes) are associated with hyperinsulinaemia. In the two studies which have investigated calpain-10 mRNA expression in human skeletal muscle, both reported a significant reduction in calpain-10 expression in subjects with the G/G genotype at SNP-43 (Baier et al., 2000; Carlsson et al., 2005). In the first of these studies, subjects with the G/G genotype had higher insulin levels at baseline which reached significance during a 2 h OGTT (Baier et al., 2000). In the second study it is not known whether the G/G subjects had higher insulin but in a separate experiment in that study, calpain-10 mRNA expression was determined in subjects with normal (NGT) and impaired glucose tolerance (IGT) before and after an insulin clamp. Interestingly, subjects with hyperinsulinaemia (IGT group) appeared to have lower levels of basal and insulin stimulated calpain-10 mRNA, although this data was not significant. When these subjects were infused with Intralipid for 24 h prior to the insulin clamp, the NGT subjects had increased calpain-10 mRNA following the insulin infusion, whereas the IGT subjects did not. These data suggest the possibility that hyperinsulinaemia may negatively regulate calpain-10 expression and

perhaps prevent a protective increase in calpain-10 mRNA in response to stimuli which induce insulin resistance. It should be noted that in that study, the authors did not detect a significant affect of the 2 h insulin clamp procedure on calpain-10 mRNA levels, but this is consistent with the data in this thesis demonstrating that up to 6 h of insulin infusion is required to downregulate calpain-10 mRNA.

It is important to put the finding that insulin downregulates calpain-10 mRNA into perspective and to consider alternative explanations. For example, insulin infusion for just 3 h has been shown to regulate the expression of approximately 800 genes in healthy human skeletal muscle (Rome et al., 2003) and has been shown to lead to an increase in the mRNA expression of other proteolytic genes, e.g. many proteasome components and ubiquitin-conjugating enzymes (Rome et al., 2003). It is generally accepted that physiological hyperinsulinaemia promotes muscle protein anabolism, possibly via inhibition of protein breakdown (Gelfand and Barrett, 1987) and/or stimulation of protein synthesis (Biolo et al., 1995). Therefore, perhaps the effect of insulin infusion on calpain-10 expression seen in this thesis reflects the potential role of calpain-10 in skeletal muscle protein turnover, rather than its proposed role in carbohydrate metabolism. This idea would be consistent with the effects of insulin infusion on the expression of other protease components (Rome et al., 2003) and would also be consistent with the role of the ubiquitous calpains in skeletal muscle, as outlined in **chapter 1 (1.7.2)**.

The results from **chapter 7** of this thesis also suggest the possibility that calpain-10 is involved in skeletal muscle protein turnover. In this chapter, there was no difference in the expression of calpain-10 between mRNA and protein between type 2 diabetic and the sedentary control subjects but calpain-10 protein was lower when compared to endurance trained control subjects. The relative increase in calpain-10 protein in the endurance trained subjects was confined to smaller calpain-10 isoforms and immunohistochemistry analysis revealed that this may be related to the muscle fibre type. Staining of skeletal muscle for calpain-10 revealed an interesting pattern of calpain-10 distribution and demonstrated regions of more intense staining which appeared to correspond to different fibre types. Endurance trained subjects tend to have a greater proportion of type I, or slow oxidative fibres (Andersson et al., 2000) whereas the skeletal muscle from obese and type 2 diabetic subjects has been shown to contain a reduced proportion of type I fibres (Nyholm et al., 1997). Therefore, differences in calpain-10 expression between diabetic and trained subjects may be related to the muscle fibre type composition. Whilst more investigation is needed to more accurately quantify calpain-10 expression in different muscle fibre types, fibre type specific expression of calpain-3 has been demonstrated in porcine skeletal muscle (Jones et al., 1999) suggesting that this may be an interesting line of future investigation.

The human *in vivo* evidence outlined in this thesis therefore suggests that the initial association between calpain-10 and diabetes is not confirmed. Critically, this highlights the difference between the presence of an environment-gene association, which might not necessarily imply a cause and effect relationship, and a functional environment-gene interaction under conditions of altered nutrient supply and insulin sensitivity.

For example, one of the initial studies on calpain-10 showed that lower skeletal muscle calpain-10 expression in Pima Indians with the risk SNP-43 genotype was associated with reduced rates of insulin stimulated glucose disposal and carbohydrate oxidation (Baier et al., 2001). This finding, however, does not prove that calpain-10 expression is either the cause of these metabolic alterations or the result of them – instead it merely demonstrates a statistical association between the two. It is possible that the lower levels of calpain-10 in these subjects was completely unrelated to their metabolic characteristics and instead was the result of an alternative factor. This factor could be the SNP-43 genotype *per se*, for example. In other words, the SNP-43 genotype may well reduce calpain-10 transcription but this does not necessarily mean that this is the cause of the reduction in carbohydrate utilisation in these subjects. This thesis extends the work from these association studies and demonstrates that in healthy human subjects, skeletal muscle calpain-10 expression is not important in states of altered nutrient supply and insulin sensitivity and

therefore provides strong evidence against a role for calpain-10 in the physiological adaptations that occur following these interventions.

One of the major limitations of this thesis was that analysis of calpain-10 was limited to the mRNA and protein expression only and the measurements in this thesis do not provide any insight into the potential post-translational roles of calpain-10. These may include specific proteolytic activity towards a number of substrates that are important for skeletal muscle metabolism. It is possible that altered calpain-10 activity, rather than expression, underlie the association of calpain-10 with insulin resistance and type 2 diabetes. This is impossible to rule out in the present thesis without parallel measurements of calpain-10 activity in the studies in this thesis. The activity of calpain-10 has not been measured in any of the previous literature and it is not clear whether calpain-10 is proteolytically active at all. Looking at its protein structure (**chapter 1, 1.8**) it would appear that the active site triad found in the ubiquitous calpains is intact in calpain-10, indicating the potential for calpain-10 to demonstrate proteolytic activity. However, it lacks the typical calcium binding sites of μ - and m-calpain indicating that any potential activity may not be regulated by intracellular calcium availability.

The potential proteolytic substrates for calpain-10 are unknown but one strong candidate in the literature is the specific glucose transporter, GLUT4, as any interaction between GLUT4 and calpain-10 might be

expected to have a significant impact on glucose uptake into cells. GLUT4 can be cleaved by m-calpain *in vitro* (Otani et al., 2004) and specific inhibition of calpain-10 reduces insulin stimulated translocation of GLUT4 to the plasma membrane in adipocytes (Paul et al., 2003). The expression of GLUT4 was measured in chapters 3 and 5 in an attempt to detect an interaction between GLUT4 and calpain-10. In chapter 3, short term fasting reduced GLUT4 mRNA significantly as insulin sensitivity was reduced but had no effect on calpain-10 mRNA or protein expression suggesting a divergence between these two proteins. Similarly, in chapter 5, insulin infusion downregulated calpain-10 mRNA but did not regulate GLUT4 mRNA levels. A potential drawback of this analysis is that GLUT4 expression may not be an ideal marker for insulin resistance and it is likely that GLUT4 translocation plays a more direct role in determining insulin mediated glucose uptake into skeletal muscle. Calpain-10 may mediate GLUT4 translocation only indirectly via effects on the insulin stimulated actin reorganisation required for GLUT4 translocation, although this effect has only been demonstrated in adipocytes *in vitro* (Paul et al., 2003). There is therefore little evidence currently to suggest that calpain-10 plays a direct role in GLUT4 pathways of glucose uptake into skeletal muscle.

It therefore follows that if calpain-10 is involved in insulin resistance and type 2 diabetes via proteolytic modification of key protein substrates, then what are these potential substrates aside from GLUT4 that are important in the relevant pathways? It was outlined in chapter 1 that

calpains are capable of cleaving a number of proteins involved in a wide range of cellular processes ranging from gene transcription to skeletal muscle cell differentiation. One interesting but speculative candidate is the signalling intermediate PKC as this has previously been shown to be cleaved by calpains *in vitro* (Kishimoto et al., 1983, 1989). A role for calpain-10 in PKC related pathways may link calpain-10 to insulin stimulated GLUT4 translocation and FFA mediated pathways of insulin resistance. Other speculative candidates may be the proteins that are involved in vesicle fusion and docking and which may be important in GLUT4 exocytosis. It has previously been shown that an isoform of calpain-10 binds to a number of proteins (e.g. SNAP-25, VAMP-2 and syntaxin-1) important for insulin-containing vesicle fusion in pancreatic cells *in vitro* (Marshall et al., 2005) but this has yet to be demonstrated in muscle cells. Recent data from the calpastatin overexpressing mice also suggests that CaMKII and AMPK may be calpain substrates as the protein content of these was increased in the skeletal muscle with calpain inhibition (Otani et al., 2006). Pathways utilising these signalling intermediates may link calpain, and possibly calpain-10, with exercise mediated glucose uptake although the data from chapter 6 of this thesis suggests that calpain-10 and -3 may not be important in such pathways.

An additional limitation in this thesis is that calpain-10 expression in skeletal muscle was examined in skeletal muscle only and this is just one of the key tissues in carbohydrate and fat metabolism. Therefore

the results in this thesis do not exclude a role for calpain-10 in the pancreas and in the adipocyte, two important tissues in the development of diabetes. Indeed *in vitro* studies have demonstrated a potential role for calpain-10 in these tissues as discussed in **chapter 1 (1.8.2)** and as a result a “two-hit” model whereby calpain-10 expression in the pancreas and insulin sensitive tissues (muscle and adipose tissue) together could affect glucose utilisation has been proposed. In addition, some interesting novel data from chapter 7 in the present thesis suggests that calpain-10 expression is highly localised to the skeletal muscle microvasculature and it is possible that a role for calpain-10 in this tissue underlies the association of calpain-10 and insulin resistance. More *in vitro* and *in vivo* work is needed to better understand these potential models.

9 Reflections and future work

In summary, based on the present observations and on reflections on the previously published literature on calpain-10, it appears unlikely at this point that calpain-10 is involved in skeletal muscle insulin resistance. A number of key questions regarding the function of calpain-10 and -3 in skeletal muscle, however, still remain. Much more is known about calpain-3, but problems with the purification are hindering detailed studies into its activation and substrate specificity. It has been shown that calpain-3 is calcium sensitive and it is clearly capable of self-autolysis, but the significance of this autolysis is not clear. Its location in skeletal muscle and the fact that loss of function mutations in its gene lead to LGMD2A indicates that calpain-3 must play a critical functional role in skeletal muscle, but how does a lack of calpain-3 activity lead to muscular dystrophy? It seems likely that its function is more in keeping with the traditional calpains and that it is important for skeletal muscle protein turnover. Its effects on LGMD2A would certainly indicate this. Whether its activity is also important for glucose utilisation pathways however, remains to be seen, but the results in this thesis would indicate otherwise.

Clearly, much less is known about calpain-10. To date, the purification of the full length protein has not been possible due to its almost complete insolubility and this is clearly an important step. Moreover, it is not known whether it is calcium activated, or indeed whether it

possesses any activity at all. The regulation of its expression and the functional significance of the observed calpain-10 isoforms also are not clear. Much speculation has surrounded calpain-10 since the initial publication but elucidating the mechanisms whereby it may affect insulin resistance and type 2 diabetes has been an immense challenge. Future work must first focus on the calpain-10 protein *in vitro* and attempt to decipher its complex regulation of expression, its mechanism of activation and its specific substrates, if indeed there are any. Calpain-10 KO mice have been produced and the phenotypic effects of this KO are currently being investigated and these mice will undoubtedly yield novel and very important data. Future human studies need to be performed with larger number of subjects to allow for detailed investigations into the complex role of genetic variation in the calpain-10 gene and of potential nutrient gene interactions.

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Appendix 1

Determination of serum insulin concentration

For the determination of serum insulin, polypropylene tubes coated with anti-insulin antibodies were incubated overnight with 200 µl of serum or standard and 1000 µl of ¹²⁵I-insulin. Supernatants were then decanted and left over tissue paper for 2-3 minutes to remove all remaining liquid. The tubes were then counted in a γ -radioactivity counter (Cobra II auto-gamma-counter, Canberra-Packard, Melbourne, Australia), and results were calculated from a standard curve of known concentrations of insulin.

Determination of plasma FFA

In a 96-well plate, 4 µl of sample was added to each well in duplicate, and for the standard curve, 0-9 µl of a 1 mM oleic acid solution was added to each well in duplicate to give a standard concentration range of 0-0.0291 mM FFA. Following this, 75 µl of colour reagent A was added to each well before incubating the plate at 37°C for 10 min. Next, 150 µl of reagent B was added to each well and the plate was incubated for a further 10 min at 37°C. The absorbance of each sample and standard was measured at 550 nm. To calculate the FFA

concentration of each unknown sample, the standard curve was plotted and used to obtain the FFA concentration in each well, and this figure was multiplied by 61 to account for the dilution of the sample in the well by the other reagents used throughout the assay.

Determination of blood ketones (β -hydroxybutyrate)

This assay was based on that described by Williamson et al. (1962) and was performed by Sally Cordon at the School of Biomedical Sciences, University of Nottingham. Briefly, 150 μ l of whole blood was added to 300 μ l of 10% perchloric acid, mixed and allowed to precipitate. The sample was then centrifuged at 1000 g for 5 minutes and the supernatant was removed to a fresh tube and the sample was frozen at -20°C. A set of standards ranging from 0 – 100 μ M was prepared in advance in the same way as the samples (i.e. PCA precipitation). Samples were neutralised with the addition of 110 μ l of 20% KOH and the pH of the resulting supernatant was checked with pH indicator paper. A pH of 7-8 was optimal. The samples were then centrifuged for 5 minutes at 1000 g. The standards were neutralised with 65 μ l of 20% KOH and 45 μ l of H₂O (to make up the volume to 110 μ l) and processed in the same way as the samples. Either 100 μ l of sample or standard was added to a well in a 96-well plate in triplicate and 55 μ l of nicotinamide-adenine dinucleotide (NAD) solution (200 μ l of NAD solution with 2 ml of hydrazine tris buffer) was added to each well. The NAD (2 mg) was reconstituted in 200 μ l. The hydrazine tris buffer was

prepared by the addition of 2 mg of EDTA to 100 μ l hydrazine hydrate, 500 μ l of 1 N HCl and 1.4 ml 0.1 M tris buffer (pH 8.5). A plate reader was set to 30°C and the plate was read at 340 nm. One microlitre of enzyme was then added to each well except one of each standard, which served as a sample blank. The plate was incubated at 30°C for 1 h and read at 340 nm. To calculate the absorbance change during the reaction for the standards, the mean first read values were subtracted from the mean final read values. The absorbance change was plotted against β -hydroxybutyrate concentration and this was used as the standard curve. The concentration of the sample was calculated in the same way from the standard curve.

Determination of glucagon concentrations

This assay was performed by Dr. K. Chokkalingam at the Queens Medical Centre, Nottingham. Briefly, 200 μ l of each sample and supplied standard were transferred to an individual glass tube and 100 μ l of glucagon antiserum was added. Tubes were vortexed and incubated at 4°C for 24 h. The next day 100 μ l of 125 I glucagon was added to each tube and the samples were again left for 24 h at 4°C. The following day, 1 ml of cold precipitating solution was added to the tubes and the samples were centrifuged at 1500 g for 15 minutes. The supernatant was decanted and the tubes were counted for 1 min (Cobra auto-gamma-counter).

Determination of urine urea concentrations

This assay was performed by Dr. K. Chokkalingam at the Queens Medical Centre, Nottingham. Urine urea was quantified using a commercially available kit (Randox Laboratories, UK). Briefly, urine samples were diluted 10-fold and 2 μ l of sample was added to 200 μ l of reagent A in well of a 96-well plate in duplicate. Serial 2-fold dilutions of the provided standard solution (13.3 mM) were prepared and 2 μ l were also added to 200 μ l of reagent A in a well of a 96-well plate in duplicate. The plate was incubated at 37°C for 30 min and then read at 340 nm.

Appendix 2

Table A.1 Real-time PCR primers and probes used in this thesis

Gene	Accession no.	Primer/Probe	Sequence
Calpain-10	NM_023083	Forward primer	5'-GTGCCTTGCAGGGAGACTCT-3'
		Taqman probe	5' (FAM)-TGTTCTGGCTCCCCTTACTGGAAAAGGTCTAC-(TAMRA) 3'
		Reverse primer	5'-CGTAGGACCCATGGACCTTG-3'
GLUT4	NM_001042	Forward primer	5'-GCTGTGGCTGGTTTCTCCAA-3'
		Taqman probe	5' (FAM)-CAACTTCATCATTGGCATGGGTTTCCA-(TAMRA) 3'
		Reverse primer	5'-CCCATAGCCTCCGCAACATA-3'
Calpain-3	NM_000070	Forward primer	5'-GGCGGAAGGACCGGAAGCT -3'
		Taqman probe	5' (FAM)-TCGCCATCTACGAGGTTCCCAAAGAGAT-(TAMRA) 3'
		Reverse primer	5'-TCCTTCTGCAGGTGCTGC T-3'
PDK4	NM_002612	Forward primer	5'-CAAGGATGCTCTGTGATCAGTATTATTT-3'
		Taqman probe	5' (FAM)- CATCTCCAGAATTAAGCTTACACAAGTGAATGGA-(TAMRA) 3'
		Reverse primer	5'-TGTGAATTGGTTGGTCTGGA-3'
PDK2	NM_002611	Forward primer	5'-CATCATGAAAGAGATCAACCTGCTT-3'
		Taqman probe	5' (FAM)- CCGACCGAGTGCTGAGCACACCC-(TAMRA) 3'
		Reverse primer	5'-CAGGAGGCTCTGGACATACCA-3'
α-Actin	NM_001100	Forward primer	5'-GAGCGTGGCTACTCCTTCGT-3'
		Taqman probe	5' (FAM)-ACCACAGCTGAGCGCGAGATCGT-(TAMRA) 3'
		Reverse primer	5'-GTAGCACAGCTTCTCCTTGATGTC-3'

Table A.2 Real-time PCR primers and probes used in this thesis (contd)

Gene	Accession no.	Primer/Probe	Sequence
HKII	NM_000189	Forward primer	5'-AAGTTCTTGTCTCAGATTGAGAGTGACT-3'
		Taqman probe	5' (FAM)-CTGCAACACTTAGGGCTTGAGAGCACCTG-(TAMRA) 3'
		Reverse primer	5'-CAGTGCACACCTCCTTAACAATG-3'
PGC1α	NM_013261	Forward primer	5'-GGTGCAGTGACCAATCAGAAATAA-3'
		Taqman probe	5' (FAM)-ATCCAATCAGTACAACAATGAGCCTTCAAACATAT-(TAMRA) 3'
		Reverse primer	5'-TTGCCTCATTCTCTTCATCTATCTTC-3'